Universidade de Aveiro Departamento de Química
2018

RITA PINHEIRO LOPES

PRODUÇÃO DE IOGURTE SOB ALTA PRESSÃO – EFEITO NO PROCESSO FERMENTATIVO E NAS CARACTERÍSTICAS DO IOGURTE

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Química Sustentável, realizada sob a orientação científica do Doutor Jorge Manuel Alexandre Saraiva, Investigador Auxiliar do Departamento de Química da Universidade de Aveiro; da Doutora Ivonne Delgadillo Giraldo, Professora Associada com Agregação do Departamento de Química da Universidade de Aveiro e da Doutora Ana Maria Pereira Gomes, Professora Associada da Escola Superior de Biotecnologia da Universidade Católica Portuguesa.

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Dedico este trabalho aos meus pais e ao André por sempre acreditarem em mim.

o júri

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agradecimentos

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palavras-chave

Fermentação, iogurte, *stress*, adaptação, alta pressão, temperatura.

resumo

Recentemente, as fermentações sob condições não convencionais têm vindo a ganhar destaque na literatura, devido às possíveis melhorias que podem trazer para os processos fermentativos. Por exemplo, quando aplicada a fermentações alimentares, esta abordagem pode resultar em novas características tanto do processo fermentativo, como também dos géneros alimentícios produzidos. Desta forma, a produção de iogurte foi utilizada, neste trabalho, como um caso de estudo, onde o efeito da variação da pressão (10-100 MPa) e da temperatura (25-50 ºC) durante a fermentação foi estudado.

Numa fase inicial, foi realizado um estudo preliminar para desenvolver estratégias para reduzir o tempo e os recursos experimentais no decorrer do trabalho. De seguida, a produção de ácido láctico e o consumo de lactose foram analisados ao longo do tempo de fermentação, de modo a estudar a cinética do processo de fermentação. Verificou-se que o consumo de substrato e formação de produtos são muito dependentes das condições utilizadas na produção de iogurte, com o aumento da pressão a diminuir a velocidade de fermentação, com a fermentação a ser mais rápida a 43 ºC. No entanto, as fermentações a 10 MPa apresentaram os resultados mais interessantes (do ponto de vista da velocidade do processo), uma vez que os perfis fermentativos foram semelhantes ao do controlo (fermentação à pressão atmosférica, 0.1 MPa) para todas as temperaturas testadas. Mais especificamente, a fermentação a 10 MPa/43 ºC correspondeu às condições ótimas para a produção de iogurte, onde o rendimento e a eficiência de produção de ácido láctico foram melhorados relativamente à fermentação a 0.1 MPa – rendimento de 1.40 $g_P\ g_S^{-1}$ e eficiência de 75 % a 10 MPa *versus* 0.79 $g_P\ g_S^{-1}$ e 40 % a 0.1 MPa, respetivamente.

Para além disso, algumas características dos iogurtes finais foram também avaliadas e foram detetadas diferenças entre eles. No caso das bactérias ácido-lácticas, o seu crescimento durante o processo fermentativo foi afetado pelas condições de fermentação, resultando em cargas microbianas diferentes. Por exemplo, foram observadas cargas microbianas superiores a 8.00 $\log_{10}$ (CFU g^{-1}) no final das fermentações a 35 e 43 ºC, enquanto que nas fermentações a 50 ºC apenas foram observadas cargas microbianas entre 3.00 e 6.50 $\log_{10}$ (CFU g^{-1}). Comparando as duas bactérias starter, *Streptococcus thermophilus* foi mais sensível à combinação de altas pressões e temperaturas do que *Lactobacillus bulgaricus*. Quanto às propriedades físicas do iogurte, a variação das condições fermentativas teve impacto tanto na sinérese como na textura dos iogurtes. Os iogurtes fermentados a 10 MPa apresentaram as características mais semelhantes aos iogurtes controlo, sendo caracterizados por níveis semelhantes de sinérese e uma textura firme sem ser excessiva.

De modo a comparar os metabolitos presentes nos diferentes iogurtes produzidos, foi também realizado um estudo metabolómico. Neste estudo,

foram detetadas várias diferenças na acumulação de metabolitos correspondentes a produtos da fermentação responsável pela produção de iogurte, tais como compostos aromáticos, ácidos orgânicos, álcoois, entre outros. No entanto, a principal diferença foi verificada nos compostos responsáveis pelo aroma amanteigado do iogurte, visto que quantidades superiores de diacetilo foram detetadas nos iogurtes produzidos a 0.1 MPa, enquanto que maiores quantidades de acetoína foram detetadas nos iogurtes produzidos sob pressão. Estas diferenças podem refletir-se na perceção sensorial dos iogurtes, podendo traduzir-se num sabor mais suave nos iogurtes produzidos a 10 MPa.

Em suma, a variação da pressão e temperatura durante a fermentação de diferentes alimentos pode ser utilizada não só para regular a velocidade fermentativa do processo, mas também para produzir um género alimentício com características diferentes. Desta forma, os processos fermentativos podem ser adaptados de modo a melhorar a qualidade alimentar e expandir as escolhas dos consumidores. Este trabalho abre então a possibilidade de aplicar este tipo de abordagens a uma grande variedade de processos fermentativos alimentares, podendo assim ajudar na produção e desenvolvimento de novos produtos alimentares.

keywords Fermentation, yogurt, stress, adaptation, high pressure, temperature.

abstract

Fermentation under non-conventional conditions has gained prominence in the last years, due to the possible process improvements. Food fermentation under sub-lethal pressures is one of such cases, and may bring novel characteristics and features not only to fermentative processes, but also to the final food products. In this work, yogurt production was used as a case-study of this approach and the effect of variation of both pressure (10-100 MPa) and temperature (25-50 ºC) during fermentation was studied.

Initially, a preliminary study was performed aiming the development of strategies to reduce the experimental time and resources during the work. Then, a kinetic study was conducted, evaluating lactic acid production and lactose consumption over fermentation time. Fermentative rates were highly dependent on the fermentation conditions used, with the increase of pressure slowing down yogurt fermentation and higher rates achieved at 43 ºC. But, interesting features were obtained at 10 MPa, where pH variation profiles were similar to those of atmospheric pressure (0.1 MPa) at almost all temperatures tested. In particular, fermentation at 10 MPa/43 ºC presented the optimal conditions, where yield and efficiency of lactic acid production during fermentation were improved relatively to fermentation at 0.1 MPa – 1.40 $g_P\ g_S^{-1}$ of yield and 75 % of efficiency at 10 MPa against 0.79 $g_P\ g_S^{-1}$ and 40 % at 0.1 MPa, respectively.

In addition, the final yogurts produced were analyzed regarding their microbiological and physical properties, and differences were observed between yogurts. In the case of starter cultures, it was found that their growth is affected by the fermentation conditions used during yogurt production: fermentations at 35 and 43 ºC lead to final microbial counts higher than 8.00 $\log_{10}$ (CFU g^{-1}), while counts between 3.00 and 6.50 $\log_{10}$ (CFU g^{-1}) were obtained after the fermentations at 50 ºC. Comparing both starter cultures, *Streptococcus thermophilus* was more sensitive to the combination of high temperature and high pressure than *Lactobacillus bulgaricus*. Regarding physical properties of the gel network, both syneresis and texture were influenced by the variations of the fermentation conditions. In this case, the yogurts fermented at 10 MPa presented characteristics more similar to the yogurts produced at 0.1 MPa (syneresis levels similar to control samples and a firm texture without being excessive).

A comparative metabolomic study was performed to analyze the metabolites present on the yogurts produced. Several differences were observed in the metabolite accumulation, including aromatic compounds, organic acids and alcohols, all products of yogurt fermentation. The main difference was verified on the compounds responsible for butter-like flavor in yogurt, with diacetyl being present in higher amounts on 0.1 MPa yogurts, while higher amounts of

acetoin were obtained on 10 MPa yogurts. These differences can be reflected in the sensorial perception of yogurts, where 10 MPa yogurts can present a softer flavor than yogurts produced at 0.1 MPa.

Overall, variation of pressure and temperature during food fermentations can be used not only to regulate the fermentation kinetics, but also to produce a final product with different characteristics. As a consequence, the processes can be modulated to improve food quality and expand the consumer choices of the fermented product. Therefore, this work opens the possibility of applying these type of strategies on a wide range of food fermentative processes, with potential to create and develop new food products.

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List of Publications

The current thesis is based on the following scientific papers:

Mota, M.J., Lopes, R.P., Koubaa, M., Roohinejad, S., Barba, F.J., Delgadillo, I., Saraiva, J.A., 2018. Fermentation at non-conventional conditions in food-and bio-sciences by the application of advanced processing technologies. Crit. Rev. Biotechnol. 38, 122–140. ..... CHAPTER II

Lopes, R.P., Mota, M.J., Sousa, S., Gomes, A.M. Delgadillo, I., Saraiva, J.A., 2018. Combined effect of pressure and temperature for yogurt production. Food Res. Int. (submitted)..... CHAPTER V

Lopes, R.P., Mota, M.J., Pinto, C.A., Sousa, S., Gomes, A.M. Delgadillo, I., Saraiva, J.A., 2019. Physicochemical and microbial changes in yogurts produced under different conditions of pressure and temperature. LWT - Food Sci. Technol. 99, 423-430. .....CHAPTER VI

During my studies I have also contributed to the following scientific productions which are not included in this thesis:

Lopes, R. P.; Mota, M. J.; Gomes, A. M.; Delgadillo, I.; Saraiva, J. A., 2018. Application of high pressure with homogenization, temperature, carbon dioxide, and cold plasma for the inactivation of bacterial spores: a review. Compr. Rev. Food Sci. F. 17, 532-555.

Neto, R.; Mota, M. J.; Lopes, R. P.; Delgadillo, I.; Saraiva, J. A., 2016. Growth and metabolism of *Oenococcus oeni* for malolactic fermentation under pressure. Lett. Appl. Microbiol. 63, 426-433.

Mota, M. J.; Lopes, R. P.; Delgadillo, I.; Saraiva, J. A., 2015. Probiotic yogurt production under high pressure and the possible use of pressure as an on/off switch to stop/start fermentation. Process Biochem. 50, 906-911.

Roselló-Soto, E.; Koubaa, M.; Moubarik, A.; Lopes, R. P.; Saraiva, J. A.; Boussetta, N.; Grimi, N.; Barba, F. J., 2015. Emerging opportunities for the effective valorization of wastes and by-products generated during olive oil production process: Nonconventional methods for the recovery of high-added value compounds. Trends Food Sci. Technol. 45, 296-310.

Santos, M. D.; Queirós, R. P.; Fidalgo, L. G.; Inácio, R. S.; Lopes, R. P.; Mota, M. J.; Sousa, S. G.; Delgadillo, I.; Saraiva, J. A., 2015. Preservation of a highly perishable food, watermelon juice, at and above room temperature under mild pressure (hyperbaric storage) as an alternative to refrigeration. LWT - Food Sci. Technol. 62, 901-905.

Queirós, R. P.: Santos, M. D.; Fidalgo, L. G.; Mota, M. J.; Lopes, R. P.; Inácio, R. S.; Delgadillo, I.; Saraiva, J. A., 2014. Hyperbaric storage of melon juice at and above room temperature and comparison with storage at atmospheric pressure and refrigeration. Food Chem. 147, 209-214.

Fidalgo, L. G.; Santos, M. D.; Queirós, R. P.; Inácio, R. S.; Mota, M. J.; Lopes, R. P.; Gonçalves, M. S.; Neto, R. F.; Saraiva, J. A., 2014. Hyperbaric storage at and above room temperature of a highly perishable food. Food Bioprocess Technol. 7, 2028-2037.

List of Abbreviations

ACE	angiotensin-I-converting enzymes
ADP	adenosine diphosphate
ATP	adenosine triphosphate
CoA	coenzyme A
DNA	deoxyribonucleic acid
DNS	3,5-dinitrosalicylic acid
Dpp	di-and tripeptides transporter
DR	diacetyl reductase
EF	electric fields
EMP	Embden–Meyerhof–Parnas
EPS	extracellular polysaccharides
FDR	false discovery rate
FID	free induction decay
GC-MS	gas chromatography–mass spectrometry
GRAS	generally recognized as safe
HP	high pressure
HPLC	high-performance liquid chromatography
HSPs	heat-shock proteins
LAB	lactic acid bacteria
LC-MS	liquid chromatography–mass spectrometry
LDH	lactate dehydrogenase
NADH/NAD^+	nicotinamide adenine dinucleotide
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NMR	nuclear magnetic resonance
Opp	oligopeptide transporter
PCA	principal component analysis
PEP-PTS	phosphoenolpyruvate-dependent phosphotransferase system
PLS-DA	partial least squares discriminant analysis
RI	refraction index
RT	room temperature
UDP	uridine diphosphate
UHT	ultra-high temperature
US	ultrasound
UV	ultraviolet

TSP-*d4*	3-(trimethylsilyl)propionic-2,2,3,3-*d4* acid

CHAPTER I

Yogurt and lactic acid bacteria:
A literature revision

1.1. Introduction

Fermentation was one of the first methods practiced by humans for milk transformation into products with an extended shelf-life, called fermented dairy products. These products are usually prepared using lactic acid bacteria (LAB) starter cultures in a controlled fermentation. In this way, LAB use nutrients in milk to support their growth and the subsequent production of lactate reduces the pH of these products, inhibiting the growth of many pathogenic and spoilage microorganisms (Gilliland, 1991; Hui et al., 2012; Tamime and Robinson, 1999).

One of the most popular dairy products is yogurt. Its origin is not well defined, but it is believed that its beneficial influence on human health and nutrition has existed in many civilizations over a long period of time. For instance, Ilya Ilyich Metchnikoff, the Nobel prize winner in Medicine in 1908, postulated that LAB involved in yogurt fermentation suppress the putrefactive-type fermentations of the intestinal flora and the consumption of these yogurts played a role in maintaining health (Soccol et al., 2010). In fact, the first mass production of yogurt was started by the pharmacist Isaac Carasso, with the goal of combating intestinal infections (Kalantzopoulos, 1997). Therefore, the production was initially confined to natural yogurt, being the market limited, in large measure, to those who believed in the health benefits of this dairy product. However, attitudes towards yogurt consumption gradually changed and, in the 1950s, new types of fermented milks arise, prepared by addition of fruits or flavoring, enrichment with vitamins or addition of selected probiotic bacteria, such as *Lactobacillus acidophilus* and several *Bifidobacterium* species (Kurmann, 1984; Puhan, 1988; Zourari et al., 1992). Subsequently, an entirely fresh image was given to yogurt and it became a popular and inexpensive snack food or dessert (Tamime and Robinson, 1999).

Nowadays, in many modern societies, fermented dairy products make up a substantial proportion of the total daily food consumption. Therefore, yogurt has gained a global prominence and economic importance across the planet (Figure 1.1), with the yogurt consumption steadily increasing worldwide over the last years (Adolfsson et al., 2004; Penna et al., 2006). For instance, 46.47 million tons of fresh dairy products were consumed in 2015 by the European Union residents, forecasted to grow to a volume of 48.42 million tons in 2025 (Statistica, 2016).

By definition, yogurt is a coagulated milk product that results from fermentation of lactose in milk by the thermophilic LAB *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, which live together symbiotically. Thus, a carefully selected mixture of LAB species is needed to complement each other and also to achieve a remarkable efficiency in acid production (Adolfsson et al., 2004; Belitz et al., 2009; Bourlioux and Pochart, 1988). In order to be called a "live and active culture yogurt", the finished yogurt product must contain live LAB in amounts $\geq 7 \log_{10}$ (CFU g^{-1}) at the time of manufacture, and the cultures must remain active at the end of the stated shelf-life (WHO/FAO, 2003).

Figure 1.1. Yogurt consumption by citizens of 15 countries each year. The numbers refer to the amount of yogurt cups (1 cup = 125 g) (DANONE Nutrition Research, 2013).

Acidification of milk during fermentation leads to the disruption of the internal structural properties of casein micelles present in milk due to the solubilization of colloidal calcium phosphate (Dalgleish and Law, 1989). As caseins approach their isoelectric point (pH 4.6), the net negative charge on caseins is reduced, decreasing the electrostatic repulsion between casein molecules and increasing the casein-casein attractions due to the increase of hydrophobic and electrostatic interactions (Horne, 1998; Lucey, 2004). In consequence, the aggregation of casein proteins and its precipitation occur and, at the same time, the whey is trapped (Haque et al., 2001; Hui et al., 2012; Rawson and Marshall, 1997). Therefore, the acidification process results in a product with a three-dimensional network consisting of caseins clusters and chains (Mulvihill et al., 1995), a gel-like texture and characteristic tang (due to lactate production) (Bender, 2006; Gilliland, 1991; Haque et al., 2001).

1.1.1. Industrial production

The production methods of yogurt have, in essence, changed little over the years. There was only some refinements regarding to LAB used in order to improve fermentation and/or produce yogurts with novel features (Tamime and Robinson, 1999). However, the manufacturing methods, raw materials, and formulations vary widely from country to country, resulting in products with a diversity of flavor and texture characteristics. For example, yogurt is produced from cow milk in many Western societies, but other mammalian milks can also be used to produce yogurt (Hui et al., 2012). For instance, sheep, goat and buffalo milks are very popular to produce yogurt in countries

around the Mediterranean, Middle Eastern countries, southern Russia and the Indian subcontinent (Tamime and Robinson, 1999).

Nowadays, there are two main types of yogurt: set and stirred yogurt, being the correspondent manufacturing procedures represented in Figure 1.2. Fermentative process of set yogurt occurs at the retail pots resulting in a continuous gel structure in the consumer container. On the other hand, the acid gel formation of stirred yogurt occurs in large fermentation tanks, where is disrupted by agitation (stirring) and pumped through a screen, which gives a smooth and viscous texture to the resulting product. Only afterwards stirred yogurt is transferred to the consumer containers (Lee and Lucey, 2010; Tamime and Robinson, 1999). Despite these differences, the main production steps of these two types of yogurt are the same, including standardization of milk (fat and protein content), homogenization, milk heat treatment, incubation/fermentation, cooling, and storage (Figure 1.2).

In the beginning of the process, milk is subjected to several pretreatment operations before culture incubation in order to create the growing conditions for bacteria culture and to improve the yogurt appearance and consistency (Figueiredo et al., 2001). Initially, milk is mixed with skim milk and cream to standardize (or adjust) the fat content to the desired level (0.5–3.5 % fat). Additionally, in this step, the solids-non-fat content (12.5 %) is also standardized with addition of nonfat milk powder, which improves the body and decreases the syneresis, which is the expelling of the interstitial liquid due to the protein molecules association and shrinkage of gel network, being undesirable to yogurt (Tamime and Robinson, 1999). Stabilizers (e.g., pectin or gelatin) are also often added to milk base in order to enhance or maintain the appropriate yogurt properties, including texture, mouthfeel, appearance, viscosity/consistency and also to prevent the whey separation (wheying-off) (Tamime and Robinson, 1999). The use of stabilizers may help to provide a more uniform consistency and lessen batch to batch variation, but some textural defects such as over-stabilization and under-stabilization may occur (Lee and Lucey, 2010).

Prior to culture addition, milk is pasteurized, which also influences the physical properties and microstructure of yogurt, improving the gel stability and decreasing the syneresis (Lucey et al., 1998a, 1998c, 1998b; Tamime and Robinson, 1999). Additionally, this processing step is used to destroy pathogenic microorganisms present in milk, providing therefore less competition for the starter culture. Several temperature/time combinations are used in yogurt industry. More commonly are used temperatures of 85 ºC for 30 min or 90-95 ºC for 5 min (Tamime and Robinson, 1999), but very high temperature and short time (100 °C to 130 ºC for 4 to 16 s) and ultra-high temperature (UHT – 140 ºC for 4 to 16 s) may also be used (Sodini et al., 2004).

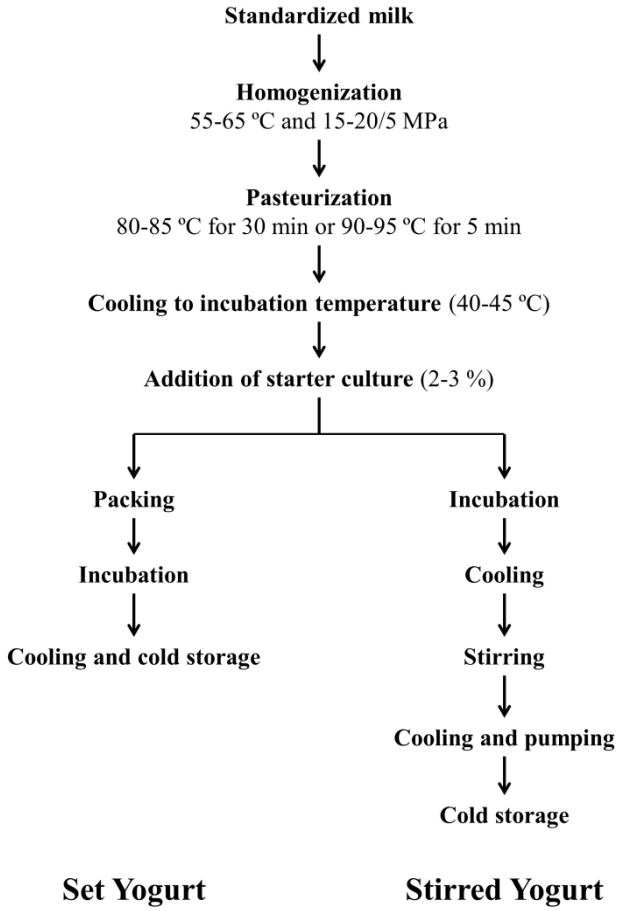

Figure 1.2. Main processing steps in the manufacture of set and stirred yogurt. Reprinted from Lee and Lucey (2010) with permission of Creative Commons Attribution License 4.0 (Copyright © 2010 Lee and Lucey).

After the heat treatment, milk is cooled to incubation temperature used for the starter culture growth, being an optimum temperature for *S. thermophilus* and *L. bulgaricus* around 40-45 ºC. The ratio between the starter cultures must be optimized depending on the cultures type used for yogurt manufacture in order to enhance flavor, acid level, and texture of the final products (Chandan and O'Rell, 2006a). During fermentation, bacteria convert lactose into lactate, reducing pH of milk (from 6.7 to ≤4.6), which in turn leads to aggregation of casein micelles and gel formation (Lee and Lucey, 2010). After yogurts reached approximately the pH 4.6, they are cooled to <10 ºC in order to inhibit the bacteria growth and its enzyme activity and to maintain the desired pH, body, and texture of the final product (Chandan and O'Rell, 2006b; Tamime and Robinson, 1999). This cooling process can be carried out using two different approaches: i) the coagulum is directly cooled to <10 ºC prior to the addition of fruit or flavoring ingredients; or ii) firstly, the coagulum is cooled to about 20 ºC for addition of fruit or flavoring ingredients and only afterwards is further cooled to <10 ºC (Tamime and Robinson, 1999). In addition, different yogurt types have different cooling mechanisms. For instance, set yogurts are directly transferred to a cold store or blast chilled in cooling tunnels, while

the stirred yogurts are first cooled by agitation in the fermentation tank, being then sheared and smoothened by devices, such as back-pressure valves, high shear devices or sieves (Lee and Lucey, 2010).

Additionally to set and stirred yogurts, there are others yogurt types commercialized, such as liquid yogurt, probiotic yogurts and strained yogurt, among others. For liquid yogurt production, clot is mechanically broken before being cooled and bottled, inducing considerable changes on the rheological properties (Thamer and Penna, 2006). On the other hand, probiotic yogurts have probiotic cultures (e.g., *L. acidophilus* and *Bifidobacterium bifidum*) in addition to yogurt cultures, which are claimed to enhance the growth of beneficial bacteria in intestine and thus its consumption is beneficial for the consumer (Bender, 2006). Strained yogurts also have great popularity worldwide, commonly called Greek style yogurt and is prepared by removing some of the whey by straining through a cloth or by centrifugation (Bender, 2006).

Hence, there are several yogurt types, which can be divided into different groups based on different characteristics, such as physical nature, chemical composition or fat content, post-fermentation processing and flavors (Tamime and Robinson, 1999), as schematized in Figure 1.3.

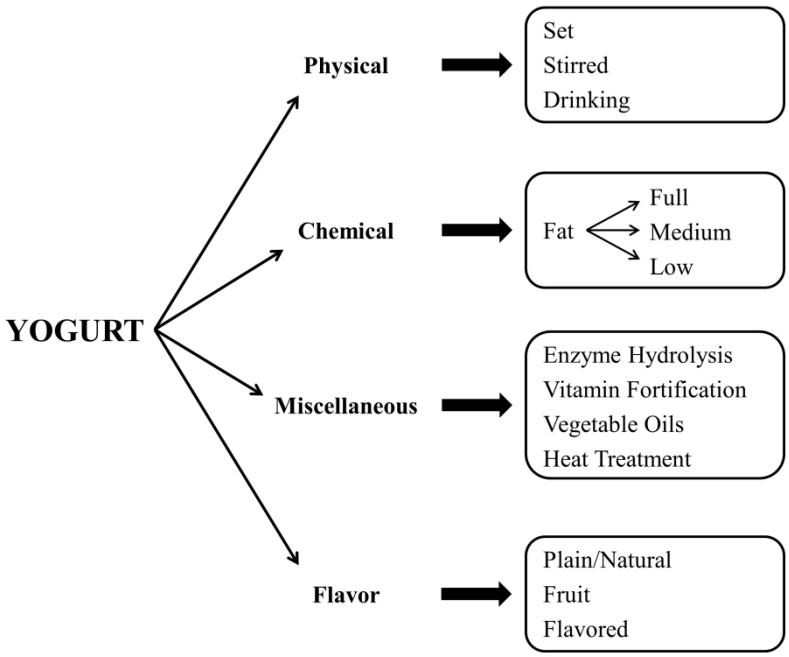

Figure 1.3. Generalized scheme for yogurt classification. Reprinted from Tamime and Robinson (1999) with permission from Elsevier.

1.2. LAB characteristics and metabolism

Yogurt starter culture is composed by thermophilic lactic acid bacteria *L. bulgaricus* and *S. thermophilus*, which were originally described by Orla-Jensen (1919). *S. thermophilus* can be exclusively isolated from the dairy environment and have some typical attributes that distinguish it from other lactic acid bacteria. For instance, while *S. thermophilus* are characterized by a spherical to ovoid shape with irregular segments, *L. bulgaricus* are rod with rounded ends shape. The optimum growth temperature is also different (37 ºC for *S. thermophilus* and 45 ºC for *L. bulgaricus*), but they grow well in cooperation at the yogurt incubation temperature (43 ºC). In common, both bacteria are able to ferment the same carbohydrates (including lactose, glucose, sucrose, fructose and sometimes galactose), and are Gram-positive, facultative anaerobic, non-motile and non-spore-forming bacteria (Chandan and O'Rell, 2006a; Hardie, 1986; Kandler and Weiss, 1986).

The role of streptococci and lactobacilli in yogurt manufacture can be summarized as milk acidification, synthesis of aromatic compounds, and development of texture and viscosity (Zourari et al., 1992). For instance, both catabolic and anabolic pathways are important for yogurt production since, on one hand, flavor and texture are improved and, on the other hand, texture-modifying polysaccharides and compounds with preservative and health-promoting properties are also produced. Therefore, to understand the metabolic reactions that occur during the fermentative process and the factors that affect the bacteria growth are fundamental to the production of a high quality yogurt (Tamime and Robinson, 1999). For this reason, the LAB metabolism is described in the next sections.

1.2.1. Associative growth of LAB

The growth association between *S. thermophilus* and *L. bulgaricus* during yogurt production used to be termed a symbiosis and this relationship has been reported by many workers, being the earliest record performed by Orla-Jensen (1931). This association could be briefly described as a positive interaction between both organisms, since leads to stimulation of each other growth and acid production, which is much larger than the sum of the acid produced by the respective single cultures (Driessen et al., 1982; Veringa et al., 1968). Therefore, this symbiotic interaction is called "proto-cooperation", since they are mutually beneficial during fermentation (Sieuwerts et al., 2008; Tamime, 2003).

In fact, the streptococci benefit from the stronger activity of the lactobacilli and in return provide certain metabolites that stimulate the growth of *L. bulgaricus* and, consequently, the acid production is remarkedly enhanced (Tamime and Robinson, 1999). Mixed yogurt cultures may also stimulate the production of some metabolites, such as acetaldehyde (Bottazzi et al., 1973; Hamdan et al., 1971), and may influence the carbohydrate utilization. For example, a *L. bulgaricus* strain that cannot use

galactose in pure culture metabolizes this sugar when it is associated with a *S. thermophilus* strain (Amoroso et al., 1989; Amoroso and De Nadra, 1988). In addition, *L. bulgaricus* has an important proteolytic activity and hydrolyzes milk proteins into small peptides and amino acids, which enhance *S. thermophilus* growth that has a limited proteolytic activity. On the other hand, *S. thermophilus* produces formate, removes oxygen from the milk, and produces carbon dioxide due to the urease activity, which, in turn, stimulates *L. bulgaricus* growth (Chandan and O'Rell, 2006a; Hui et al., 2012). In conclusion, there is the release of stimulatory factors by each starter culture, showing the associative growth of these two microorganisms (Tamime and Robinson, 1999).

1.2.2. Carbohydrate metabolism

Firstly, uptake of lactose by cells is an important factor that influences the carbohydrate metabolism. For that, LAB relies on two different transportation systems: a specific permease system or a phosphoenolpyruvate-dependent phosphotransferase system (PEP-PTS) (de Vos and Vaughan, 1994; Thompson, 1987). While the specific permease system involves cytoplasmic proteins that translocate lactose without any chemical modification, lactose is phosphorylated during the translocation into the cell in the PEP-PTS (McKay et al., 1969).

Usually, *S. thermophilus*, *L. bulgaricus*, *L. acidophilus* and *Bifidobacterium* spp. use the permease system for lactose uptake and the unphosphorylated disaccharide is hydrolyzed by a cytoplasmic β-galactosidase into non-phosphorylated β-D-glucose and β-D-galactose. Then, while glucose is catabolized via Embden–Meyerhof–Parnas (EMP) pathway (Figure 1.4), the galactose is secreted from the cell. However, when all the glucose is depleted, galactose is transported by a highly galactose-specific permease and converted to α-D-galactose by a galactose mutarotase in order to be catabolized via Leloir pathway (also represented in Figure 1.4). In this pathway, α-D-galactose is transformed to glucose-1-P that is converted to glucose-6-P, which in turn enters in the EMP pathway. Then, in the end, lactate is obtained as final product, which is secreted from the cell by a permease (Bras et al., 1991; Collins and Thompson, 1992; Daryaei et al., 2010; Hickey et al., 1986; Hutkins et al., 1985; Hutkins and Ponne, 1991; Kandler, 1983; Poolman et al., 1989; Poolman, 1993; Poolman et al., 1995, 1990; Thompson, 1988; Wu et al., 2015; Zourari et al., 1992).

On the other hand, PEP-PTS is used by lactococci and certain strains of *L. acidophilus* (Kanatani and Oshimura, 1994; Marshall and Tamime, 1997a). In this case, the resultant lactose-6-P is hydrolyzed by a 6-phospho-β-galactosidase into glucose and galactose-6-P. Here, glucose is also catabolized via EMP pathway, while the isomerization of galactose-6-P into tagatose-6-P by a galactose-6-P isomerase occur and follow the Tagatose pathway (Figure 1.4). However, the dephosphorylation of galactose may not take place, remaining unmetabolized and being subsequently excreted from the microbial cell and be only used when glucose is depleted. In both pathways, the glucose and galactose converge at dihydroxyacetone phosphate and glyceraldehyde-3-phosphate

where the three-carbon sugars become further oxidized to phosphoenolpyruvate and then produced lactate (Cogan and Accolas, 1995; Marshall and Tamime, 1997b; Tamime and Robinson, 1999; Wu et al., 2015).

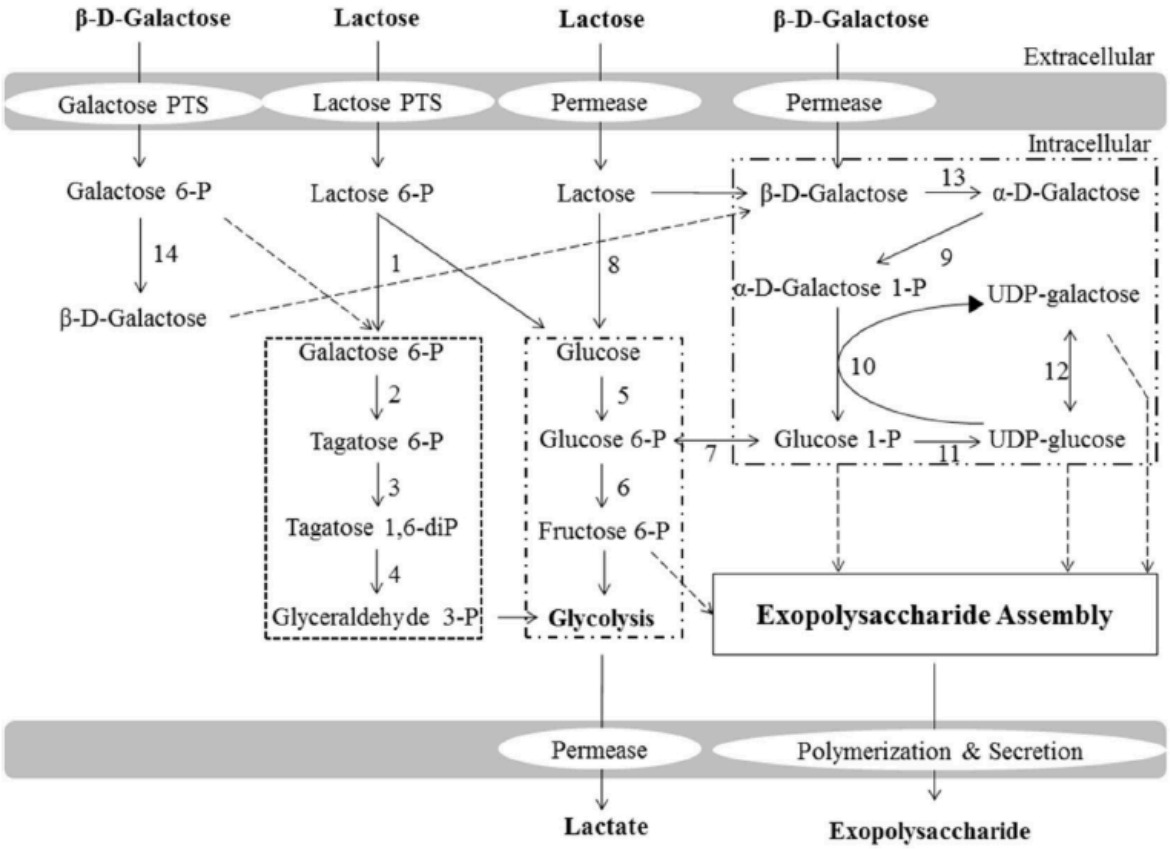

Figure 1.4. General pathways of lactose/galactose metabolism in LAB, including the Tagatose-6P pathway (square dot), Embden-Meyerhof-Parnas (EMP) pathway (dash dot), and Leloir pathway (long dash dot dot). The enzymes involved in the pathways are numbered: (1) 6-phospho-β-galactosidase, (2) galactose 6-phosphate isomerase, (3) tagatose 6-phosphate kinase, (4) tagatose 1,6-di-phospahate aldolase, (5) glucokinase, (6) phosphoglucose isomerase, (7) phosphoglucomutase, (8) β-galactosidase, (9) galactokinase, (10) galactose 1-phosphate-uridylyltransferase, (11) UDP-glucose pyrophosphorylase, (12) UDP-galactose 4-epimerase, (13) galactose mutarotase, and (14) phosphatase. Reprinted from Wu et al. (2015) with permission from Elsevier.

In addition, three types of metabolism may characterize the fermentation by LAB depending on the species: i) homofermentative metabolism, where only lactate is produced; ii) mixed acid metabolism, which corresponds to a homofermentative metabolism where formate, acetate, ethanol, and/or CO_2 can be produced in addition to lactate under certain growth conditions (described in section 2.2.2); and iii) heterofermentative metabolism, including pentose pathways which results in

the production of lactate, ethanol, acetate and CO_2 (Valenzuela et al., 2015). Both homo and heterofermentative characteristic reactions are represented in Figure 1.5. Regarding the heterofermentative metabolism, only bifidobacterial are able to perform this fermentation type, since the enzymes aldolase and glucose-6-phosphate dehydrogenase are absent in these species. Thus, hexoses are catabolized by a fructose-6-P shunt and the pathway involves a fructose-6-P phosphoketolase, resulting in the production of lactate and acetate as final products of fermentation by *Bifidobacterium* spp. (Tamime and Robinson, 1999).

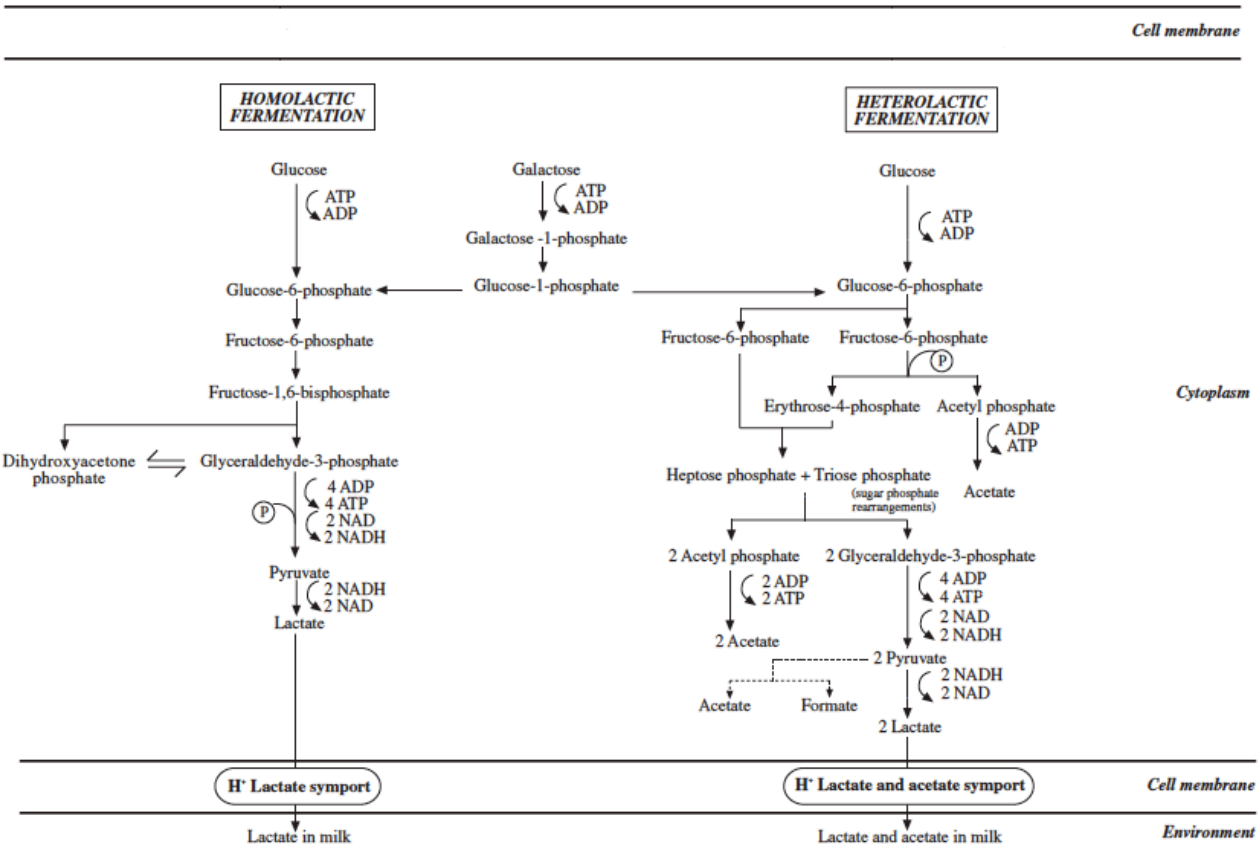

Figure 1.5. Homofermentative and heterofermentative metabolism of lactose by LAB after being hydrolyzed into glucose and galactose. Reprinted from Tamime and Robinson (1999) with permission from Elsevier.

Overall, the main product of carbohydrate metabolism of LAB is lactate, yielding 95 % of the fermentation output (Chandan and O'Rell, 2006a). Both lactate isomers are produced during yogurt production since starter cultures possess 2 distinctive lactate dehydrogenases (LDH). While *S. thermophilus* possesses 2 fructose 1,6-bisphosphate-independent L-LDH which produce mainly L-(+)-lactate (Garvie, 1978; Hemme et al., 1981), *L. bulgaricus* possesses an NAD-dependent stereospecific LDH that produces D.(-)-lactate (Gasser, 1970). On the other hand, *S. thermophilus* grows faster than *L. bulgaricus*, thus L-(+)-lactate is firstly produced followed by D-(-)-lactate (Tamime and Robinson, 1999). In addition, the L-(+)-lactate is usually present in yogurt at higher

amounts than D-(-)-lactate, usually representing 50-70 % of the total lactate (Chandan and O'Rell, 2006a).

1.2.2.1.Production of exopolysaccharides

In addition to their primary role, certain LAB strains make a further contribution to the physical structure of yogurt (Rohm and Kovac, 1994; Vlahopoulou and Bell, 1993) by production of extracellular polysaccharides (EPS). In stirred yogurt, yogurt beverages and low milk solids yogurts, EPS production can enhance some rheological properties (i.e., minimize the syneresis, improve the viscosity and texture, and modify the structure) and the sensory perception (i.e., firmness and creaminess) (Haque et al., 2001; Tamime, 2003; Zourari et al., 1992). However, it is difficult to establish a good correlation between the quantity of EPS produced and the corresponding viscosity (Olsen, 1989).

EPS consist in long-chain polysaccharides composed by branched and repeating units of sugars or sugar derivates and the most common sugars are glucose, galactose and rhamnose, present in different ratios (De Vuyst and Degeest, 1999; Welman and Maddox, 2003). Based on their composition, EPS can be divided into two different types: homo-EPS and hetero-EPS. Homo-EPS are composed by only one type of monosaccharides, being the most common the glucans and fructans containing glucose and fructose, respectively. On the other hand, the hetero-EPS are composed by repeating units of at least two types of monosaccharides or other molecules, such as glucose, galactose, rhamnose and fucose (Boels et al., 2001). In addition, EPS may also be classified according to their position in LAB. For instance, EPS can be cell wall-associated polysaccharides, being known as capsular polysaccharides (CPS), or be directly secreted from cell, being known as ropy EPS (Ruas-Madiedo and De Los Reyes-Gavilán, 2005). Therefore, different EPS types with different chemical composition, molecular size and structure may have different biofunctionalities (Ruas-Madiedo et al., 2009; Welman and Maddox, 2003).

According to Boels et al. (2001), sugars present in milk can be used as substrates for the EPS biosynthesis. In fact, lactose and galactose metabolism performed by mesophilic and thermophilic LAB (including *Lactococcus lactis*, *S. thermophilus* and *L. bulgaricus*) are closely associated with EPS production. For instance, both EMP and Leloir pathways are able to provide the priming substrates (e.g., fructose-6P, glucose-6P and galactose-1P) for the synthesis of nucleotide sugars (e.g, UDP-glucose and UDP-galactose), needed for EPS assembly as represented in Figure 1.4. On the other hand, tagatose pathway appears to not contribute to EPS production, being only important to provide substrate for the EMP pathway (Wu et al., 2015).

1.2.2.2.Production of flavor compounds

In addition, starter cultures are also primarily responsible for the production of flavor compounds that contribute to the sensorial properties of yogurt. Both volatile and non-volatile compounds present in milk and specific compounds produced during fermentation are responsible for yogurt aroma and flavor, including non-volatile acids (such as lactate, pyruvate, oxalate or succinate), volatile acids (such as formate, acetate, propionate or butyrate) and carbonyl compounds (such as acetaldehyde, acetone, acetoin or diacetyl). However, there are other compounds that could be associated, perhaps indirectly, with flavor enhancement, or act as precursors for the formation of the major aroma compounds in yogurts, including certain amino acids (derived from proteolysis and described in section 1.2.3), volatile fatty acids (derived from lypolisis and described in section 1.2.4), products of thermal degradation of milk constituents, among others (Cheng, 2010; Ott et al., 1997; Smid and Kleerebezem, 2014; Tamime and Robinson, 1999; Thierry et al., 2015).

In this section, only the production of flavor compounds by carbohydrate metabolism will be described, which use pyruvate as a metabolic precursor. As mentioned above, under certain conditions (e.g., carbon limitation, carbon excess of slowly metabolized sugars, aerobic conditions), the starter cultures may shift the homofermentative metabolism to a mixed acid metabolism with production of several metabolites, including several aroma compounds or aroma precursors (Figure 1.6) (De Felipe et al., 1998; Puri et al., 2014; Tanaka et al., 2002).

The typical aroma of yogurt is mainly characterized by acetaldehyde, with concentrations ranging from 2.0 to 4.1 mg kg^{-1} depending on the strains and process factors used during fermentation (Chaves et al., 2002; Sandine et al., 1972). For instance, the presence of specific enzymes able to catalyze the carbonyl compounds formation from the different milk constituents is preponderant for acetaldehyde formation. In fact, pyruvate can be transformed into acetaldehyde by action of pyruvate decarboxylase (reaction 6 in Figure 1.6) (Keenan and Bills, 1968; Seneca, 1955); or by pyruvate dehydrogenase to produce acetyl-CoA, which, in turn, can be reduced by an acetaldehyde dehydrogenase and produce acetaldehyde (reactions 4 and 1a in Figure 1.6, respectively) (Lees and Jago, 1977, 1976).

However, other end products may be produced in significant proportions from pyruvate (as represented in Figure 1.6), when low concentrations of lactose or glucose are present in milk. Formate, acetate, ethanol and acetoin are some of these end products (Walstra et al., 2005).

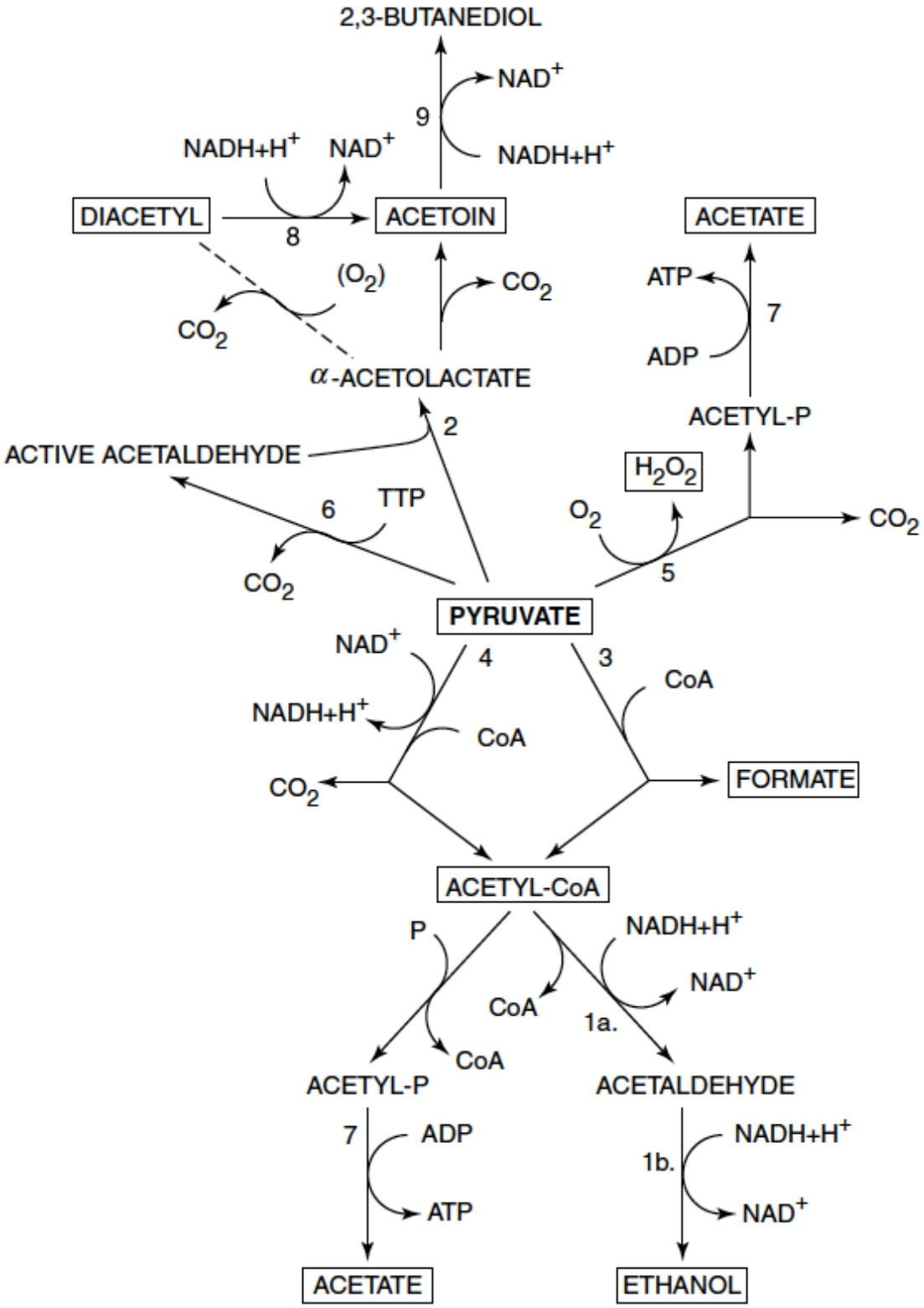

Figure 1.6. Mixed acid metabolism by LAB, showing the alternative pathways for pyruvate catabolism with formation of the main flavor compounds of yogurt. Dashed arrow denotes a nonenzymatic reaction. Important metabolites and end products are framed. Selected enzymatic reactions are numbered: (1a) acetaldehyde dehydrogenase, (1b) alcohol dehydrogenase, (2) acetolactate synthase, (3) pyruvate formate lyase, (4) pyruvate

dehydrogenase, (5) pyruvate oxidase, (6) pyruvate decarboxylase, (7) acetate kinase, (8) diacetyl reductase, and (9) acetoin reductase. Adapted from Walstra et al. (2005) with permission of Taylor & Francis Group LLC in the format Thesis/Dissertation via Copyright Clearance Center.

The alternative route for pyruvate metabolism starts with the conversion of pyruvate to acetyl-CoA and formate through action of pyruvate formate lyase (reaction 3 in Figure 1.6). Alternatively, pyruvate may be also oxidized by the pyruvate dehydrogenase pathway, being acetyl-CoA and CO_2 formed and NAD^+ reduced to NADH (reaction 4 in Figure 1.6). However, in certain thermophilic streptococci, the pyruvate formate lyase enzyme is active even when there is an excess of sugar amount and therefore formate production by *S. thermophilus* occurs. On the other hand, acetyl-CoA can be converted either to acetate via acetyl-P to generate additional ATP (reaction 7 in Figure 1.6) or to ethanol via acetaldehyde to redress the NAD^+/NADH imbalance caused by alcohol dehydrogenase (reaction 1a and 1b in Figure 1.6). In the presence of oxygen, pyruvate can be converted to acetyl-P by a pyruvate oxidase, which is in turn hydrolyzed into acetate and ATP by acetate kinase (reaction 5 and 7 in Figure 1.6, respectively) (Walstra et al., 2005).

In addition, some compounds with 4 carbon atoms (known as C_4 compounds) are also important for the typical aroma of yogurt, being responsible for the butter-like flavor. These compounds include diacetyl, acetoin and 2,3-butanediol and they can be generated from glycolysis or citrate metabolism (Chen et al., 2017; Neves et al., 2005). In the case of citrate metabolism, citrate is transported into the cell by a citrate permease and then is hydrolyzed into acetate, CO_2 and pyruvate by citrate lyase (Walstra et al., 2005). Therefore, higher production of C_4 compounds is observed in these cases, due to the additional pyruvate production from citrate metabolism.

Among the C_4 compounds, diacetyl is the most important flavor compound, being present at concentrations ranging from 0.2 to 3 mg kg^{-1} (Smith and Hui, 2015). Regarding its production, α-acetolactate is firstly produced by condensation of active acetaldehyde and another molecule of pyruvate by action of a acetolactate synthase (reaction 2 in Figure 1.6). Then, α-acetolactate can be nonenzymatically decarboxylated to acetoin or, in the presence of oxygen, oxidatively decarboxylated to diacetyl (Figure 1.6) (Walstra et al., 2005). Both *S. thermophilus* and *L. bulgaricus* are able to produce diacetyl, however *diacetylactis* strains produce higher amounts of diacetyl due to their capacity to perform citrate metabolism (Passerini et al., 2013). Acetoin is another C_4 compound, which corresponds to the reduced form of diacetyl, produced by the action of diacetyl reductase (reaction 8 in Figure 1.6). This compound is usually used for the reduction of the diacetyl harshness, contributing to the mild creamy flavor, since acetoin has a weaker flavor when comparing to the buttery flavor of diacetyl (Cheng, 2010; Walstra et al., 2005). In addition, 2,3-butanediol is the reduced form of acetoin (formed by the action of acetoin reductase – reaction 9 in Figure 1.6), making only a limited contribution to the creamy and buttery flavor of yogurt (Hugenholtz, 1993; Walstra et al., 2005).

Overall, carbohydrate metabolism by LAB is important not only for the production of lactic acid, but also to the production of several other compounds that are responsible for the texture and flavor of yogurt. In fact, the metabolic pathways of LAB are very complex, corresponding to formation of a great variety of metabolites.

1.2.3. Protein metabolism

Aggregation of proteins during yogurt fermentation is determinant for the coagulum formation and contributes markedly to the yogurt consistency (Chandan and O'Rell, 2006a). On the other hand, the proteolytic activity of yogurt strains is also important not only for the nutrition and interactions between the yogurt bacteria, but also for the production of some flavor compounds during yogurt fermentation (Chen et al., 2017; Zourari et al., 1992). For instance, the enzymatic hydrolysis of milk proteins results in production of peptides and free amino acids that can affect the gel formation and yogurt physical structure, on one hand; and, on the other hand, are also essential to the *S. thermophilus* growth (Tamime and Robinson, 1999).

Despite the peptides and amino acids produced may not contribute directly to the yogurt flavor, they act as precursors of the flavor compounds production, including ammonia, amines, aldehydes, phenols, indole, and alcohol (Chen et al., 2017; Tamime and Robinson, 1999). In fact, both proteolysis and amino acid degradation are involved in the production of these precursors. Initially, the casein proteolysis by LAB starts with the action of cell-envelope proteinases that degrade the proteins into oligopeptides (Broadbent et al., 2011). Then, the resultant di-, tri-, and oligopeptides are transported into the cell by oligopeptide transporters and they are further hydrolyzed to amino acids by peptidases present in the cytoplasm, as represented in Figure 1.7. These amino acids are then converted to α-keto acids by transamination, and they can further converted by different enzymatic reactions to produce several flavor compounds that may contribute to yogurt flavor. Reduction of α-keto acids generating flavorless α-hydroxy acids, decarboxylation of α-keto acids producing aldehydes that can be further reduced to alcohols, and α-keto acids oxidative decarboxylation forming acyl-CoA and then carboxylic acids are some of the enzymatic reactions involved in the production of flavor compounds from amino acids (Aleksandrzak-Piekarczyk et al., 2010). In addition, esters or thio-esters can be also produced during this process, through reactions between the formed alcohols and carboxylic acids by the action of esterases or acyltransferases (Smit et al., 2005).

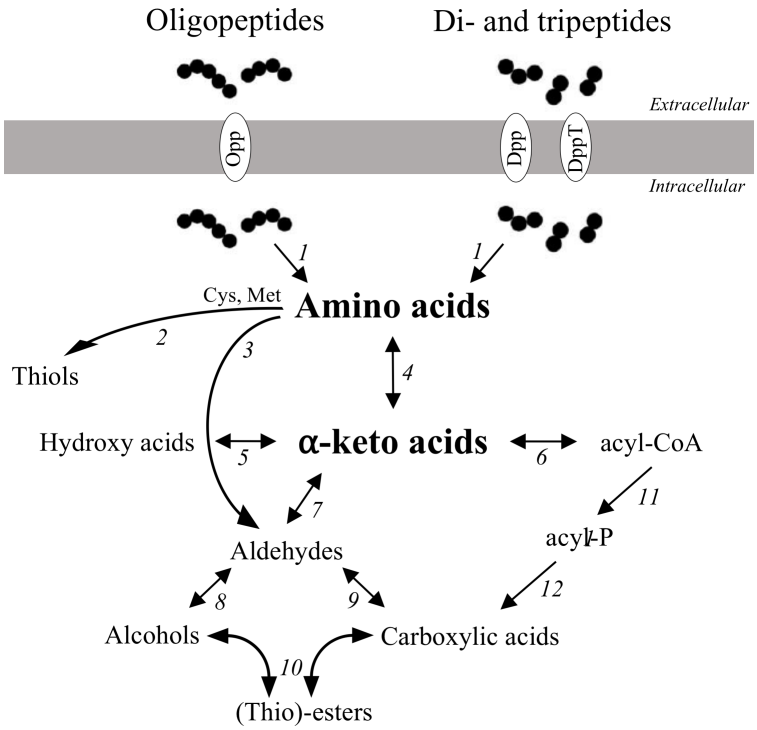

Figure 1.7. General pathways of protein metabolism in LAB relevant for flavor production. The enzymes involved in the pathways are numbered: (1) peptidase, (2) lyase, (3) aldolase, (4) aminotransferase, (5) hydroxyacid dehydrogenase, (6) α-keto acid dehydrogenase complex, (7) α-keto acid decarboxylase, (8) alcohol dehydrogenase, (9) aldehyde dehydrogenase, (10) esterase A, (11) phosphotransacylase, and (12) acyl kinase. Opp: oligopeptide transporter; Dpp, ATP-driven transporter for di-and tripeptides; DtpT, ion-linked transporter for di-and tripeptides. Adapted from Chen et al. (2017) with permission of Taylor & Francis Group LLC in the format Thesis/Dissertation via Copyright Clearance Center.

In addition, amino acids may also be converted to the respective thiols by lyases (such as, cystathionine β- and γ-lyases). For instance, methionine can be converted to methanethiol, which can be in turn converted by oxidation to dimethyl sulphide, dimethyl disulphide and dimethyl trisulphide, or by esterase-catalysed reactions to thioesters (Bustos et al., 2011). However, although these compounds are more important to cheese sensory properties, some of them are also identified as off-flavours of yogurt (Thierry et al., 2015). On the other hand, threonine aldolase also belongs to the lyases class and is able to catalyze the formation of acetaldehyde and glycine from threonine (Lees and Jago, 1977, 1976). In fact, threonine was found to correspond to the primary source for acetaldehyde production during yogurt fermentations (Chaves et al., 2002; Ott et al., 2000a). In addition, there are several other compounds that can be produced by these pathways and were identified as responsible for the yogurt flavor. For instance, the buttery, vanilla-like aroma of yogurt can also be related with the presence of 2,3-pentanedione. This compound is produced by threonine

metabolism and relies on two different pathways: i) uses threonine and pyruvate as direct precursors, and ii) uses pyruvate and acetate (Ott et al., 2000b).

Therefore, the proteolytic activity is mainly due to exopeptidases and peptidases that both *S. thermophilus* and *L. bulgaricus* possess (Tamime and Deeth, 1980) and a wide variety of flavor compounds can be produced from that, contributing to the characteristic flavor and aroma of yogurt.

1.2.4. Lipid/fat metabolism

Triglycerides constitute 96–98 % of the total milk lipids/fats and the remaining fraction consists of phospholipids, sterols, fat-soluble vitamins (A, D, E and K), fatty acids, waxes and squalene. Thus, the enzymatic hydrolysis of milk lipids takes place at the ester linkages by action of esterases or lipases, yielding fatty acids and glycerol. Therefore, any reduction of fat percentage, increase of the fatty acids level (free or esterified), or increase of the volatile fatty acids content in yogurt can be attributed to lipid metabolism by starter cultures (Tamime and Robinson, 1999).

In fact, lipid breakdown is also a source of aroma compounds of yogurt, since along with free fatty acids, saturated and unsaturated fatty acids are also derived from the decomposition of triglycerides and they correspond to precursors for the production of aroma compounds (Cheng, 2010; McSweeney and Sousa, 2000). For example, unsaturated fatty acids can be converted to hydroperoxides or 4- or 5-hydroxyacids by oxidation, with hydroperoxides being rapidly decomposed to hexanal or unsaturated aldehydes and 4- or 5-hydroxyacids being readily cyclized to cyclic compounds (Cheng, 2010; Guo et al., 2009). The main cyclic compounds present in yogurt are γ- and δ-lactones with 5- and 6-sided rings, respectively. Despite they have a strong fruity flavor, an excessive or unbalanced lipid oxidation and lipolysis can lead to off-flavor formation. For instance, products of lipid oxidation as aldehydes and ketones give the stale and “oxidized” flavors to dairy products (Chen et al., 2017).

However, lipolysis is generally low in yogurt since most LAB posses only intracellular esterases and thus they are only able to hydrolyze lipids after being released from lysed cell. Therefore, the difference between the free fatty acid content of yogurt and milk is only slight and subsequently, the contribution of lipolysis to the yogurt flavor is limited. (Alm, 1982; Chen et al., 2017; Rasic and Vucurovic, 1973).

1.2.5. Vitamin metabolism

The vitamin content also changes during yogurt production, influencing the vitamin available at the time of consumption of the products (Chandan and O’Rell, 2006a). For instance, vitamin content (namely, vitamin B_{12}, biotin and pantothenic acid) decreases due to i) an excess of dissolved oxygen in milk; ii) moderate/excessive heat treatment of milk; iii) a long incubation period; iv) its

consumption by yogurt starter cultures during fermentation, reducing the nutritional value of the product; and iv) yogurt storage (Chandan and O'Rell, 2006a; Tamime and Robinson, 1999). On the other hand, vitamin content also increases during fermentation, namely niacin and folate content, which are actively synthetized by starter cultures. Regarding the folate biosynthesis, its synthetic pathways for *S. thermophillus* and *L. bulgaricus* are not well established. On the other hand, niacin synthesis by *S. thermophilus* and *L. bulgaricus* may occur from the nicotinamide fraction arising during the formation of NAD^+ and/or $NADP^+$ (Tamime and Robinson, 1999). Nevertheless, yogurt corresponds to an excellent source of vitamins (Chandan and O'Rell, 2006a).

1.2.6. Production of antimicrobial compounds

Despite the symbiotic relationship between yogurt bacteria, growth inhibition is also often observed (Martins and Luchese, 1988; Moon and Reinbold, 1974; Suzuki et al., 1982). This inhibition may be due to competition for nutrients (Moon and Reinbold, 1976) or to inhibitory compounds produced by strains, such as bacteriocins and inhibitory peptides (Martins and Luchese, 1988).

Bacteriocins have been defined as "extracellularly released primary or modified products of bacterial ribosomal synthesis, which can have a relatively narrow spectrum of bactericidal activity". This activity is characterized by the inclusion of at least some strains of the same species as the producer bacterium and against which the producer strain has some mechanism(s) of specific protection (Jack et al., 1995). Additionally, it has been established that the bacteriocins target is the cytoplasmic membrane of sensitive pathogens and other bacteria (Servin, 2004). Regarding to the so-called "attack" mechanisms, it is known that the inhibitory peptide adheres to certain targets in the cell membrane (Huang, 2008; Shai, 2002).

In the case of LAB, the bacteriocins produced have received much attention in terms of food safety due to their generally recognized as safe (GRAS) status (Settanni and Corsetti, 2008). For instance, *L. bulgaricus* and *L. acidophilus* produce two different bacteriocins called bulgarican and acidophilin, respectively, which inhibit the growth of several spoilage bacteria, having a wide spectrum activity against both Gram-positive and Gram-negative bacteria (Otles and Cagindi, 2003; Reddy et al., 1984; Shahani et al., 1972). *S. thermophilus* also produces bacteriocins that have inhibitory activity towards molds from *Aspergillus* and *Rhizopus* genera (Baglio, 2014; Marciset et al., 1997). Therefore, these and other bacteriocins may inhibit the growth of spoilage and pathogenic microorganisms that are undesirable in dairy products. As a consequence, they can act as a natural preservative, increasing the shelf-life of the food product without the addition of chemical preservatives (Tamime, 2003).

1.2.7. Miscellaneous changes

Additionally, numerous other changes may occur in milk constituents during yogurt production. For instance, the content of citric and hippuric acid decreases, while the levels of acetic and succinic acid increase during yogurt fermentation. Other changes that may occur, involve i) detectable nucleotides, with the increase of adenosine monophosphate, uridin monophosphate, guanine monophosphate and adenine dinucleotide amounts; ii) minerals, whose distribution changes with the increase of the ionic forms and destabilization of calcium caseinate–phosphate complexes; iii) uracyl-4-carboxylic acid (also known as orotic acid), which is metabolized by starter cultures; iv) metal ions, which are used to stimulate the growth of starter cultures; v) β-galactosidase, proteases and peptidases, which are accumulated in the matrix; vi) 7 α-dehydroxylase activity on bile acids, which is negative for starter cultures preventing the production of secondary bile acids; vii) angiotensin-I-converting enzymes (ACE), whose inhibitory activity is low in yogurt; viii) several other enzymatic activities (superoxide dismatase, endonuclease, etc.) ; and ix) immunostimulating agent, which promotes an immune response against Gram-negative bacteria in intestine (Baglio, 2014; Tamime and Robinson, 1999).

1.3. Yogurt composition

The chemical composition of a foodstuff provides useful indication on its potential nutritional value. In Table 1.1, the main components of some typical natural and fruit yogurts are indicated. Therefore, it is evident that yogurt may be an important introduction to any diet, with the precise impact depending upon the type of yogurt being consumed (Robinson, 1977).

The nutrient composition of yogurt is mainly based on the nutrient composition of the milk from which it is derived. The milk composition is in turn affected by many factors, including genetic and individual mammalian differences, feed, stage of lactation, age, and environmental factors, such as the season of the year. However, the yogurt production process also plays an important role on the final composition of yogurt. For instance, the changes in milk constituents during lactic acid fermentation have a significant effect on the nutritional and physiologic value of the finished yogurt product. These changes depend on the species and strains of the fermentative bacteria and the temperature and duration of the fermentative process. In addition to fermentation, the temperature of milk processing, duration of heat exposure, the source and type of milk solids that may be added before fermentation, exposure to light, and storage conditions are some of other variables that can affect the final composition of yogurt (Adolfsson et al., 2004).

Table 1.1. Some typical values of the major constituents of milk and yogurt (all units 100 g^{-1}). Adapted from Holland et al. (1991) and Buttriss (1997).

Constituent	Milk		Yogurt[a]			
	Whole	Skim	Full fat	Low fat	Low fat/fruit	Greek-style
Water (g)	87.8	91.1	81.9	84.9	77.0	77.0
Energy value (kcal)	66	33	79	56	90	115
Protein (g)	3.2	3.3	5.7	5.1	4.1	6.4
Fat (g)	3.9	0.1	3.0	0.8	0.7	9.1
Carbohydrate (g)	4.8	5.0	7.8	7.5	17.9	NR
Calcium (mg)	115	120	200	190	150	150
Phosphorous (mg)	92	95	170	160	120	130
Sodium (mg)	55	55	80	83	64	NR
Potassium (mg)	140	150	280	250	210	NR
Zinc (mg)	0.4	0.4	0.7	0.6	0.5	0.5

[a] The nutrient levels in fruit yogurt will vary with the type of fruit and stabilizer.

NR: Not reported.

Sugars are present in high amounts on yogurt, including glucose, lactose, added sugars (such as, sucrose and fructose), and lactulose, a disaccharide composed by galactose and fructose that is obtained during the milk pasteurization due to epimerization of lactose. However, lactose is the predominant sugar in yogurt, present at higher concentrations than the other sugars, even after fermentation since only a reduction of ≈30 % is observed during this process (Andrews, 1984; Barrantes et al., 1994; Chandan and O'Rell, 2006a; Scrimshaw and Murray, 1988). Interestingly, the lactose present in yogurt is not able to cause an intolerance reaction in humans, since β-galactosidase is released from starter cultures promoting the intraintestinal digestion of lactose. Thus, the lactose level is too low to cause an adverse reaction, when reach the colon (Desmaison et al., 1990; Gallagher et al., 1974).

Regarding organic acids, lactate is the acid present in higher amounts (0.8 – 1.3 %). In fact, despite the lactose is in excess in the fermentative medium, lactate concentrations higher than 1.5 % acts as a growth inhibitor of yogurt starter cultures. During fermentation, both isomers are produced, with L-(+)-isomer being produced in higher amounts. In nutritional terms, the L-(+)-isomer is more easily digested than the D-(-)-isomer, since it is poorly metabolized and an excessive intake is reported to cause acidosis in some children. However, a normal consumption intake does not pose any hazard (Chandan and O'Rell, 2006a; Tamime and Robinson, 1999).

In addition, other compounds are present in lower amounts, including volatile, non-volatile and carbonyl compounds, and other compounds derived from thermal degradation of lipids, lactose and proteins during milk processing (Moon and Reinbold, 1974; Tamime and Deeth, 1980; Turcic et al., 1969). These compounds are responsible for the yogurt flavor and their concentration is determinant

for production of a yogurt with more or less pleasant and intense tastes. For instance, acetaldehyde is considered as the major flavor component of yogurt and its final concentration ranges from 2 to 40 mg kg^{-1}, while acetoin is present at 2 to 6 mg kg^{-1}, acetate at 1 to 4 mg kg^{-1} and diacetyl at 0.5 to 1.0 mg kg^{-1} (Cheng, 2010; De Noni et al., 1998; Dumont and Adda, 1973; Gaafar, 1992; Law, 1981; Laye et al., 1993; Pette and Lolkema, 1950).

Yogurt is also an attractive source of proteins and amino acids. For instance, a consumption of around 200–250 mL of yogurt per day can easily provide the minimum daily requirement of animal protein (15 g) to consumers (Altschul, 1965; Cheeseman, 1991). Additionally, yogurt protein is more easily digested than milk protein, mainly due to the proteolytic activity of starter cultures that predigest the proteins during fermentation, resulting in a higher content of peptides and free amino acids in yogurt (Beshkova et al., 1998; Rasic and Kurmann, 1978; Shahani and Chandan, 1979). On the other hand, both heat treatment and acid production during fermentation result in finer coagulation of casein, which also contributes to the improvement of protein digestibility in yogurts. In addition to casein, milk contains whey proteins that remain soluble at low pH values and contribute to approximately 20 % of the total protein content. The main whey proteins are β-lactoglobulin, α-lactalbumin, bovine serum albumin (BSA) and immunoglobulins, which constitute about 50, 20, 10 and 10 % of the total whey proteins in bovine milk, respectively (Adolfsson et al., 2004; Fox and McSweeney, 1998; Haque et al., 2001).

Other yogurt constituents are lipids, which are present in 3 – 4 g 100 g^{-1} in traditional yogurts and 9 – 10 g 100 g^{-1} in Greek-style yogurts (Buttriss, 1997). Lipids are known as the most valuable source of energy, with each gram of fat providing around 9 kcal. Since the yogurt lipolysis is low, the lipids present in yogurt are similar to the milk lipids that yogurt is derived and they are present at the same amounts. Therefore, a wide range of fatty acids characterizes the yogurt lipids, being present mostly in glyceride form as well as the milk lipids (Alm, 1982; Tamime and Robinson, 1999).

Additionally, yogurt is an excellent dietary source of minerals for human nutrition, including calcium, phosphorus, magnesium and zinc. In fact, the bioavailabilities of the minerals present in both yogurt and milk are mostly similar, with high proportions available for absorption and utilization by the body. In addition, most of the calcium and magnesium present in yogurt must be in the ionic form (due to the low pH of yogurt), which may affect positively the physiological efficiency of the minerals absorption. Thus, yogurt may also act as a source of calcium for sufferers of lactose intolerance. (Buttriss, 1997; Chandan and O'Rell, 2006a).

Vitamins are also present in yogurt, but its content depends on several factors, including the processing parameters and the subsequent storage conditions. Therefore, the relative availability of vitamins in yogurt is difficult to access, but corresponds to a source of vitamins in human diet. In fact, yogurts may be fortified with vitamins (e.g., vitamins A and C), and certain B group vitamins may be synthesized by starter cultures during fermentation process (e.g., niacin and folate). Therefore, the careful selection of the starter cultures and the processing conditions is important to

maintain the nutritional properties of yogurt (Chandan and O'Rell, 2006a; Tamime and Robinson, 1999).

1.4. Health benefits of yogurt

The nutritional value of a particular food depends on its digestibility and its content of essential nutrients. As previously mentioned, both digestibility and nutrient contents may be improved by fermentation, in which the enzymatic activity of starter culture may predigest the macronutrients. For instance, yogurt is better tolerated than milk by lactose-deficient individuals due to the release of β-galactosidase that improves the intraintestinal digestion of lactose, as explained above (Tamime and Robinson, 1999). Additionally, yogurt has been known for its nutraceutical, therapeutic, and probiotic attributes, claiming to improve digestion and bioavailability of milk constituents, inhibit the harmful bacteria of gastrointestinal tract, have immunostimulatory and anticarcinogenic activities and a hypocholesterolaemic effect (Kalantzopoulos, 1997; Mathur et al., 2000; Penna et al., 2007; Welman and Maddox, 2003).

In addition, fermented milks are claimed to contain a number of biologically active compounds which may contribute to human health. These compounds include bacteria used for fermentation, their metabolic products and components derived from milk. For instance, stimulation of the normal microflora of gut has been attributed to the regular consumption of yogurt, since the lysing cells of starter cultures release vitamins or other growth factors that may enhance the *L. acidophilus* growth in the small intestine (Robinson, 1989). However, there is a consensus among scientists that yogurt starter cultures are not able to adhere to the mucosal surfaces in the intestinal tract. Thus, recently, yogurts may also contain *L. acidophilus*, *Lactobacillus paracasei* subsp. *paracasei* and/or *Bifidobacterium* spp, which are often referred as probiotic yogurts (Marshall and Tamime, 1997b; Pedrosa et al., 1995; Speck et al., 1993). These products may be similar to yogurt in terms of chemical composition, but the microflora impact on the consumer digestive system is completely different, with health benefits exerted on the intestine (Table 1.2) (Tamime and Robinson, 1999).

Additionally, lactic acid bacteria have a strong inhibitory effect against growth and toxin production by the most of other bacteria, including spoilage organisms and pathogens that may contaminate food products. This antagonistic activity can be the result of the competition for available nutrients, decrease in redox potential, production of lactate and acetate that decrease the pH, production of other inhibitory primary metabolites (e.g., hydrogen peroxide, carbon dioxide and diacetyl), and production of special antimicrobial compounds (e.g., bacteriocins and antibiotics) (Kalantzopoulos, 1997; Kansal, 2001; Kodama, 1952). For instance, it has been claimed that LAB ingestion may counteract the effect of *Escherichia coli* outgrowth on the gut, possibly due to the

production of anti-*E. coli* metabolites, the detoxification of enterotoxins, the inhibition of toxic amine synthesis, or gut adhesion, preventing the colonization of the gastrointestinal tract by pathogenic bacteria (Shiby and Mishra, 2013). Therefore, the regular yogurt consumption present several health benefits to humans, with the live and active LAB present in yogurt playing an important role to the beneficial effects of this fermented dairy product.

Table 1.2. Some of health-promoting activities attributed to starter cultures in yogurt and probiotic yogurt, with an indication of their validity for humans. Adapted from Tamime and Robinson (1999).

Action/Effect	Alleged health benefit	Established in humans[a,b]
In digestive tract	Active against *Helicobacter pylori*	
	Enhanced lactose digestion	●
	Stimulation of intestinal immunity	
	Stabilization of Crohn's disease	
	Stimulation of intestinal peristalsis	
On intestinal microflora	Improves balance between microbial populations	Increase in faecal bifidobacteria
	Decrease of faecal enzyme activity	●
	Colonization of intestinal tract	●
	Reduced carrier time for Salmonella spp.	
On diarrhea	Prevention/treatment of acute diarrhea	●
	Prevention/treatment of rotavirus diarrhea	●
	Prevention of antibiotic-induced diarrhea	●
Other effects	Improved immunity to disease	
	Suppression of some cancers	
	Reduction of serum cholesterol	
	Reduction of hypertension	

[a] More than one publication and no conflicting evidence.

[b] A tick indicates confirmed treatments in humans.

1.5. General outlook about yogurt production

Overall, yogurt production evolved over the years, changing from crude and elementary procedures to more controlled ones. This change was only possible because of the increase of the scientific knowledge about not only the technological aspects of the process, but also the LAB physiology. In fact, the characterization and genome sequencing of the starter cultures allowed a better control of lactic acid fermentation and the improvement of the yogurt quality. Therefore, the understanding of the production process of yogurt and the LAB metabolism enable the development

of a wide range of novel products with desired properties and specific features, and make it possible to provide health benefits to consumers, by addition of probiotics, for example (Aryana and Olson, 2017; Gänzle, 2015; Tamime, 2003).

The improvement of the available technologies may lead to new research areas, involving the development of novel yogurt production procedures and the study of the yogurt role on the human health. For instance, study of the pathogens presence in these products, the incorporation of additional probiotics and functional ingredients, and the use of novel processing and packaging technologies are some of the possible directions of future research on yogurt production. The continuous improvements of yogurt with production of value-added products (i.e., with an increase of the health benefits associated to its consumption) will enhance the consumer acceptability and, as a consequence, the manufacturers profits (Aryana and Olson, 2017).

1.6. References

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CHAPTER II

Fermentation at non-conventional conditions: A literature revision

This chapter is based on information of the following publication:

Mota, M.J., Lopes, R.P., Koubaa, M., Roohinejad, S., Barba, F.J., Delgadillo, I., Saraiva, J.A., 2018. Fermentation at non-conventional conditions in food- and bio-sciences by application of advanced processing technologies. Crit. Rev. Biotechnol. 38, 122–140.

2.1. Introduction

Stress is one of the major driving forces of microbial evolution and adaptation, bringing forward new strains (Serrazanetti et al., 2009). Microorganisms may show different reactions when exposed to stress: at more extreme conditions, cells are unable to withstand and adapt, leading to microbial destruction; while, at milder stress conditions, microorganisms may be able to survive and grow, due to activation of general and specific stress response mechanisms (Huang et al., 2014; Lado and Yousef, 2002). These stress responses rely on the coordinated expression of genes that alter different cellular processes (e.g., cell division, DNA metabolism, housekeeping, membrane composition, transport, etc.) to improve the stress tolerance (Storz and Hengge, 2000; van de Guchte et al., 2002). Some of the stress-induced genes seem to be genuinely specific, while others correspond to general stress response genes that can be induced by a wide variety of stresses (De Angelis et al., 2001; Serrazanetti et al., 2009).

The exposure of microbial cells to stressful conditions during growth and fermentation involves a complex network of response mechanisms, with several metabolic activities that will reflect upon the metabolome of the fermentative microorganisms, and thus on the bioproducts and on the bioprocess itself (Serrazanetti et al., 2009). Some of the changes promoted by these stress responses may have a positive outcome, such as enhanced cell growth, improved fermentation rates and yields, or even changes in metabolic selectivity. Therefore, the concept of performing fermentations using stressful "non-conventional" conditions is arising, which is based on the use of emerging processing technologies (typically applied for food pasteurization) at sub-lethal levels, in order to affect microbial growth and fermentation, but without causing microbial inactivation. The most studied technologies in this context include electric fields (EF), ultrasound (US) and high pressure (HP). There are two main distinct approaches for the use of these emerging technologies (Figure 2.1), and both will be addressed in this chapter: the first is the application of the stress (EF, US or HP) to the inoculum, with fermentation subsequently taking place at normal conditions; while the second corresponds to application of the stress during fermentation, whether continuously during the entire process or intermittently with fixed duration pulses.

At the moment, some microorganisms were tested under these non-conventional conditions, aiming not only the stimulation of cell growth, but also the improvement of relevant fermentation processes, giving novel characteristics and features to the final products obtained. These industrially relevant processes include not only food fermentations (e.g., for the production of dairy products, alcoholic beverages, and others), but also the production of commodity bio-chemicals (e.g., acetic acid, citric acid, and ethanol) and high-value bio-products (e.g., vitamins, antibiotics, and biopolymers). This chapter will be mainly focused on the application of this approach to food fermentations. Since the application of both EF and US on food fermentation process was recently reviewed by Galván-D'Alessandro and Carciochi (2018), and the present thesis is devoted to HP, so here only the HP application will be addressed in detail.

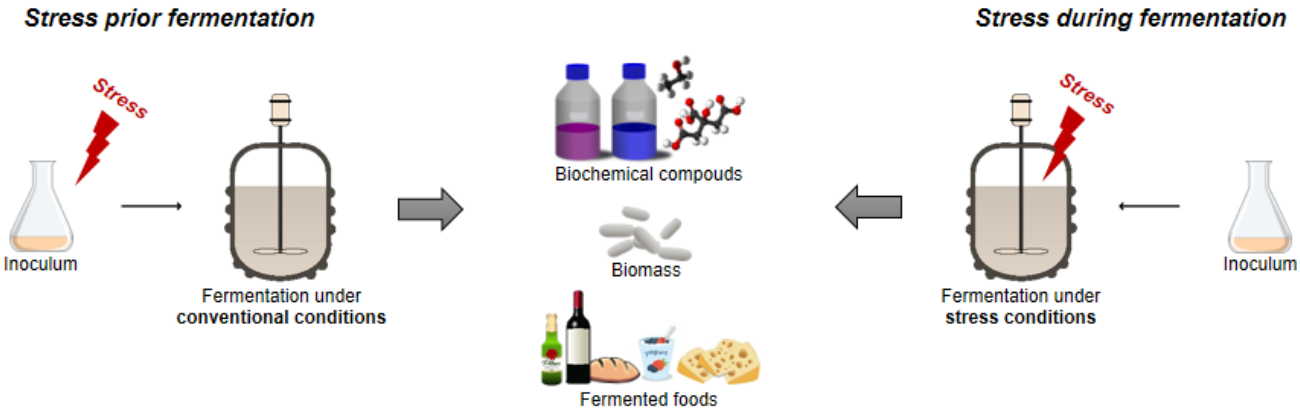

Figure 2.1. The two main approaches presented in literature for the use of emerging technologies (electric fields, ultrasound, or high pressure) to stimulate cell growth and fermentation: i) application of the stress to the inoculum, with fermentation subsequently taking place at normal conditions; ii) application of the stress during fermentation, whether continuously during the entire process or intermittently with fixed duration pulses.

2.2. High pressure application to fermentation processes

High pressure (HP) may bring novel characteristics to fermentative processes when applied at sub-lethal levels, similarly to other non-thermal technologies. When mesophilic microorganisms are exposed to HP, cell morphology and metabolism are modified accordingly. The structures and functions of cell wall and cell membrane, biochemical reactions, and gene expression can all be affected by pressure (Coelho et al., 2004), subsequently leading to a remodeled cell metabolism through either directly targeting the chemical reactions or changing the expression of relevant genes involved in these reactions (Lee et al., 2006).

Huang et al. (2014) reviewed the response of microorganisms to pressure and concluded that microorganisms are more likely to be stressed or injured than killed under HP treatment, particularly at lower intensities. In this case, microorganisms have sets of genes able to regulate environmental adaptation, which expressions generally leads to the production of heat-shock proteins (HSPs). The accumulation of these HSPs within the cell enhances the cell resistance to multiple environmental stresses (Lou and Yousef, 1997; Wemekamp-Kamphuis et al., 2004). For instance, Welch et al. (1993) reported that a HP treatment of 55 MPa induced a stress response on cells, where cold-shock proteins, heat-shock proteins, and other protective proteins were accumulated. In addition, Hörmann et al. (2006) used a comparative proteome approach to characterize the HP effects on *Lactobacillus sanfranciscensis*, concluding that HP stress response uses subsets of other stress responses (such as cold and high salinity).

The effects of HP on microbial cells are influenced by several factors including the level and duration of the pressure treatments, the compression/decompression rates and other process

parameters (temperature, media composition, pH, etc.). Moreover, each microbial strain has a specific degree of HP tolerance according to its intrinsic cellular characteristics. In general, prokaryotes are more HP-resistant than eukaryotes, Gram-positive bacteria are more HP-resistant than Gram-negative bacteria, and cocci are more HP-resistant than bacilli (Huang et al., 2014). The cell growth stage was also reported to affect the microbial tolerance to HP treatments, which is usually higher during the stationary phase than exponential phase (Huang et al., 2014).

In general, application of sub-lethal HP treatments to fermentative processes may lead to possible acquisition of new desirable characteristics, obtained by inhibition or even suppression of some metabolic pathways and/or utilization of new ones (Mota et al., 2013). This concept is gaining relevance over the last years, since piezo-tolerant strains may have numerous interesting applications in different fields (Aertsen et al., 2009; Hörmann et al., 2006). Some fermentation processes performed under sub-lethal pressures are summarized in Table 2.1.

Most studies regarding the effects of HP on microbial growth and metabolism were performed using the yeast *S. cerevisiae*. Picard et al. (2007) studied the application of sub-lethal levels of HP during the alcoholic fermentation by *S. cerevisiae*. When fermentation was performed at 5 and 10 MPa, ethanol production proceeded 3-fold faster, compared to fermentation at 0.1 MPa. This promoted the increase of fermentation yields by 6 % and 5 % at 5 and 10 MPa, respectively, relative to 0.1 MPa. However, at pressures above 20 MPa fermentation was slowed down, and they estimated that the alcoholic fermentation was interrupted at 87 ± 7 MPa. The increase of fermentative rate was suggested to be attributed to an enhancement of the activity of one or more enzymes involved in the glycolytic pathway up to 10 MPa, and with pressure increasing they are progressively repressed. These results revealed a great practical significance, mainly because of the widespread applicability of *S. cerevisiae*. In fact, the higher fermentation rate under pressure can provide a faster and more cost-effective production of alcoholic beverages, bakery products and fuel bioethanol (Mota et al., 2013). Similarly, Bravim et al. (2013) observed that pre-treatment of *S. cerevisiae* with HP led to an increase in ethanol content upon fermentation. By a global transcriptional analysis, the authors observed the over expression of several genes related to cell recovery and stress tolerance induced by HP. One of the most relevant case was the gene SYM1, where its over-expression resulted in enhanced ethanol production and stress tolerance upon fermentation. On the other hand, trehalose and glutathione are two major stress-induced metabolites with industrial value, which could be produced under HP conditions. In fact, *S. cerevisiae* CICC1339 growing at 0.5 MPa showed an increase of 58.7 % in glutathione concentration in comparison with the control cells at atmospheric pressure (Qiao et al., 2006). Similarly, application of HP at 1 MPa on *S. cerevisiae* resulted in increasing the yield of trehalose by 82.9 % (Dong et al., 2007). Trehalose protects cells through a non-specific mechanism under adverse conditions (Liang et al., 2013), while glutathione is involved in apoptosis (Kiriyama et al., 2012). These two products are normally present at very low

concentrations in microorganisms (Bachhawat et al., 2013), but their production increases when cells undergo stress, possibly for protection (Dong and Jiang, 2016).

Table 2.1. General effects of high pressure on microbial cell growth and fermentation.

Microorganism	Main effects	References
Saccharomyces cerevisiae	- Acceleration of alcoholic fermentation (up to 3-fold) - Increased ethanol yields (5-6 %) - Over expression of several genes related to cell recovery and stress tolerance, including the gene SYM1 - Increased glutathione concentration (58.7 %) - Increased trehalose yield (82.9 %)	Bravim et al. (2013); Dong et al. (2007); Picard et al. (2007); Qiao et al. (2006)
Clostridium thermocellum	- Metabolic shift, with increased ethanol production and decreased acetate (by-product) production - Higher ethanol:acetate ratio	Bothun et al. (2004)
Gluconacetobacter xylinus	- Decreased cell growth - Decreased production of bacterial cellulose - Cellulose ribbons with profound morphological differences	Kato et al. (2007)
Streptococcus thermophilus, Lactobacillus bulgaricus,* and *Bifidobacterium lactis	- Decreased fermentation rate - At higher pressures, microorganisms metabolically inhibited - Bacterial strains still viable at lower pressures, with ability to produce yogurt	Mota et al. (2015)
Oenococcus oeni	- Fermentation with *O. oeni* during and after HP-stresses - Decreased concentrations of L-lactic acid - Increased concentrations of D-lactic acid	Neto et al. (2016)

Neto et al. (2016) applied different HP-stresses (50 MPa, 8 h; 100 MPa, 8 h; 300 MPa, 0.5 h) in the beginning of fermentation with *Oenococcus oeni*, a lactic acid bacterium employed by wine industry to perform malolactic fermentation. *O. oeni* was able to grow and ferment with some metabolic changes during and after HP-stresses of 50 and 100 MPa. The HP-stress of 100 MPa resulted in lowering the concentrations of L-lactic acid and increasing the concentrations of D-lactic acid, compared to the control. In contrast, the HP-stress of 300 MPa for 0.5 h resulted in complete inactivation of *O. oeni*, but malolactic fermentation was still observed at some extent, showing that malolactic enzyme maintained some residual activity at these conditions. Although the impact of these metabolic changes in wine malolactic fermentation is still not understood, it provides important insights on the possible effects of HP on biocatalysts, which in some cases can be more resistant to pressure than the analogous microbial strain itself.

In a biotechnological point of view, some fermentations under pressure were tested with the purpose to improve the production of bio-chemicals and bio-products relevant to industry. For instance, the production of ethanol by *Clostridium thermocellum* (Bothun et al., 2004) and bacterial

cellulose by *Gluconacetobacter xylinus* (Kato et al., 2007) were studied. In the case of *C. thermocellum*, a metabolic shift towards ethanol production was observed when fermentation occurred at 7 and 17 MPa, with a 60-fold increase in the ratio ethanol:acetate when compared to atmospheric pressure. On the other hand, morphological differences were observed in the bacterial cellulose produced by *G. xylinus* under HP, with the bacterial cellulose fibers produced under HP presenting higher density than the ones produced at atmospheric pressure.

Recently, this approach begin to be applied to food fermentations, with yogurt production representing the first case-study of the application of sub-lethal pressures to these fermentations. The purpose of this case-study was to study the effect of pressure not only on the fermentative process, but also on the final product characteristics. In this case, the effect of HP (5-100 MPa) on lactic acid fermentation for the production of probiotic yogurt was studied by Mota et al. (2015), and the fermentative rate decreased with pressure increasing until no fermentation occurred at 100 MPa. However, extension of the fermentation time at 5 MPa yielded a typical pH for yogurt, indicating that the viability of the bacterial strains used (*Streptococcus thermophilus*, *Lactobacillus bulgaricus*, and *Bifidobacterium lactis*) and their ability to produce yogurt were not compromised by this pressure levels. But, more studies are needed to evaluate the HP influence on the nutritional and sensorial characteristics of the yogurt produced under pressure.

In addition, Mota et al. (2015) also verified that no fermentation was found in samples subjected to 100 MPa for 180 minutes, but these samples revealed a normal metabolic activity when they were returned to atmospheric pressure. This finding indicates that the microorganisms were metabolically inhibited (but not inactivated) during the period at 100 MPa. Taking this into account, the authors suggested the possible use of pressure as a variable to control the metabolic activity of microorganisms, similarly to how refrigeration is widely used today. Such possibility could lead to substantial energy savings, since energy is only needed to reach the intended pressure level and not to maintain it (in contrast to refrigeration, which requires energy to maintain low temperatures). This feature could be particularly important for long storage periods, reducing the associated energy costs. However, more studies are needed to understand the mechanisms behind this effect and to evaluate the feasibility of application in the process.

Application of HP in fermentative processes may have novel potentials than other non-conventional technologies, including EF and US. In other words, HP can be applied intermittently or continuously during the whole fermentation time, without seriously loss the cell (if adequate sub-lethal pressure is applied) and increase of temperature. Therefore, refrigeration is not needed, leading to a more cost effective process than the other technologies already applied in this context. Moreover, the energy costs involved in the application of HP stress during the whole fermentation process are also lower, since HP only needs energy to generate the pressure and no energy needed to maintain it. However, HP application to fermentations still present some drawbacks, since HP systems specific for fermentations are not available yet, with the equipment layouts used in literature being adapted

for a wide range of other HP experiments (such as, food pasteurization, extractions, among others). Therefore, a process optimization must be carried out in these cases to minimize the constraints caused by the low pressure range needed and the lack of agitation, aeration or temperature control of the HP equipments available nowadays. As a consequence, this process is characterized by a high equipment cost and a long experimental time for process optimization.

2.2.1. Effect of high pressure on food constituents

In addition to the effect of HP on microbial growth and fermentation, sub-lethal pressures may also cause changes on growth media during the process, which may affect the nutrient availability and subsequently the microbial growth. For instance, HP increases the solubility of gases (Averill and Eldredge, 2011), which can influence the microbial growth depending on their oxygen requirements.

On the other hand, the pressure effect on food constituents must also be taken into account when applying this approach to food fermentations. However, the information present in literature about the effect of this pressure ranges is still very scarce, since this is an emergent concept. But, it is possible to have some ideas from the effect of HP processing on food quality. Generally, this technology is gaining prominence in food industry as a mild preservation technology where the number of microorganisms are reduced and the foods shelf-life is extended, maintaining the fresh appearance, organoleptic characteristics and nutritional quality of the processed foods (Hogan et al., 2005; Torres and Velazquez, 2005). This is only possible because HP affects only non-covalent bonds (hydrogen, ionic, and hydrophobic bonds), that causes unfolding of proteins, but has little effect on chemical constituents associated with desirable food qualities, including flavor, color, or nutritional content (Hayashi, 1991). For instance, small molecules such as amino acids, vitamins, and flavor compounds are not affected by HP, while the conformation of large molecules, such as proteins, enzymes, polysaccharides, and nucleic acid, may be affected (Balci and Wilbey, 1999). As a consequence, certain product characteristics may be changed, including inhibition of the activity of certain enzymes, modification of the nutrient digestibility and bioavailability, and change of some functional properties (Aymerich et al., 2008).

In the case of proteins, HP usually causes their denaturation by destruction of hydrophobic and ionic bonds, and unfolding of molecules. Depending on the treatment conditions (pressure, temperature and time) and the protein type, the denaturation process can be reversible or irreversible (Rastogi et al., 2007). As a consequence, the unfolding proteins can interact with each other, promoting the protein aggregation and leading to formation of pressure-induced gels. Taking into account that thermal gelation is characterized by large conformational changes of proteins, the pressure-induced gels may present different mechanical properties when compared to the heat-induced gels (Cardoso et al., 2010; Truong et al., 2015). Regarding to enzymes, any conformational

change in the protein structure caused by HP, especially in the active site, can lead to activity loss or changes in the enzymes functionality (Rastogi et al., 2007).

Lipids are another main constituent of food products and lipid oxidation is one of the most important mechanisms responsible for sensorial and nutritional quality loss in food products (Kolakowska, 2002). Regarding HP effect, lipid systems are the most pressure sensitive biological components (Rivalain et al., 2010). In fact, lipid oxidation is accelerated by pressures higher than 300 MPa, with the effects being highly dependent of not only the treatment conditions, but also the composition of lipid and non-lipid fractions of the treated food (Medina-Meza et al., 2014).

Overall, the HP application may affect some food constituents, which, in turn, can change the sensorial, nutritional and functional properties of the treated food products. But, these effects are highly dependent on the treatment conditions, including pressure, temperature and time. Therefore, the effect of sub-lethal pressures on food constituents must be studied to better understand the pressure influence on food properties, when applied at this levels to food fermentations.

2.3. General outlook about the application of non-conventional conditions to fermentation processes

In addition to HP, both EF and US can also be applied at sub-lethal levels, before or during fermentation, intermittently or continuously, in order to promote stress conditions able to cause substantial changes in the cells and in the process itself. The studies currently available in literature show improvements in microbial processes, including higher cell growth, higher fermentative rates and yields, lower accumulation of by-products and/or production of different compounds. Regarding food fermentations, a final product with different characteristics can be obtained, whether at the nutritional level (bioactive compounds content) or sensorial level (texture and organoleptic properties) (Galván-D'Alessandro and Carciochi, 2018).

Despite all these technologies seem to be suitable for application during the microbial growth and fermentation (under specific and adjusted conditions), each of them has advantages and limitations, as represented in Figure 2.2. The mechanisms behind the stimulation of cell growth and fermentation by EF and US relate mainly to the increase of mass transfer and cell permeability, which increase the diffusion of nutrients and metabolites across the cell membrane, as a consequence (Galván-D'Alessandro and Carciochi, 2018; Knirsch et al., 2010; Lentacker et al., 2014; Shil et al., 2008). Thus, the process parameters must be optimized to avoid inhibition of cell growth or even cell destruction. In contrast, application of sub-lethal HP presented a higher versatility and showed some interesting distinctive features, since it can be applied as intermittent stress or continuously, without heating effects, leading to more possibilities to influence microbial processes. Despite of the strong interest of pressure application on these processes, the information in literature is still scarce, possibly

due to the low availability of HP systems and the still high cost of equipment, being these two aspects the main challenges to a more widespread study of this technology.

		Advantages	Limitations
EF		• Promote beneficial effects to fermentative processes.	• Need a careful optimization to avoid cell inhibition or destruction; • Difficult scale up; • High initial cost; • Low availability of commercial units.
US		• Well-studied technology; • Less aggressive to cells; • Low equipment costs.	• Imply the use of a refrigeration system to temperature control; • High energy requirements and high energetic costs.
HP		• Promote distinctive features to fermentative processes; • Possibility to be applied continuously without cell inactivation or heat generation.	• High initial cost; • Low availability of adapted HP systems.

Figure 2.2. Main advantages and limitations of the most studied technologies used in the context of non-conventional fermentations: EF, electric fields; US, ultrasounds; and HP, high pressure.

In general, the application of these non-conventional conditions during fermentations is still poorly explored, particularly on food fermentations. In fact, the application of HP on food fermentations can present some limitations, despite the good results obtained for lactic acid fermentations. For instance, the production of carbon dioxide during alcoholic fermentation can represent a hurdle to the application of HP because the high pressure equipments currently available are not adapted to the volume increase caused by CO_2 accumulation. But, the interesting improvements observed in the studies published so far suggest the potential development in the research field within the next years. Due to the several factors affecting the processes that still need optimization, the economic and practical feasibility of these approaches is still difficult to assess. Therefore, further studies focus on the optimization and scale up of the processes are needed, as well as the evaluation of non-conventional conditions application to other fermentative processes with relevance for both food and bio-based industries.

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CHAPTER III

Scope and outline of the thesis

Variations on the fermentation conditions (fermentative pressure, temperature, etc.) can bring novel characteristics and features not only to the fermentative processes, but also to the final products of fermentation. Thus, this can be of particular interest to the production of food fermented products with novel characteristics, such as the food nutritional and sensorial properties. Fermented dairy products are the most popular fermented products, with a substantial proportion of the total daily food consumption. For instance, yogurt has gained a global prominence and economic importance, with the production process being extensively studied and continuously improved to enhance the consumer acceptability. Therefore, yogurt is a good case-study for the application of sub-lethal levels of pressure during the fermentation process. In fact, this work belongs to a set of studies where the effect of HP on fermentative processes was extensively studied with the purpose of the production of a food product.

Thereby, the main purpose of this thesis was to study the combined effect of HP and temperature during yogurt production, in the context of fermentations under non-conventional conditions. For that, this work was divided in three different objectives:

i) Study of the combined effect of pressure and temperature on fermentation kinetics;
ii) Study of the combined effect of pressure and temperature on starter cultures growth and metabolism;
iii) Study of the combined effect of pressure and temperature on final yogurt characteristics.

Lactic acid fermentation was performed under different conditions of pressure and temperature in the course of this work, accordingly to the results obtained. In Table 3.1, the experimental plan is schematized, presenting the conditions used in each chapter.

Table 3.1. Pressure and temperature conditions used in each chapter of this thesis.

	Pressure (MPa)				**Temperature (ºC)**			
	10	30	50	100	RT[a]	35	43	50
CHAPTER IV	•						•	
CHAPTER V	•	•	•	•	•	•	•	•
CHAPTER VI	•	•				•	•	•
CHAPTER IV	•					•	•	•

[a]RT – room temperature (≈25 ºC).

In a first phase, a preliminary study was performed (**CHAPTER IV**) to verify if the procedures of decompression and compression to collect samples during the fermentation time influence the starter cultures fermentative capacity, resulting in differences on the fermentative rate. Product

formation was monitored in this case and the obtained results allow to save experience time and experimental resources in the course of this work.

In **CHAPTER V**, the combined effect of pressure and temperature on the fermentation kinetics was studied. Fermentation was performed under different combinations of pressure and temperature and the rates of acidification and substrate consumption of the starter cultures present in yogurt were measured. Taking into account the results obtained, 9 different conditions of pressure and temperature were selected to analyze the characteristics of the resultant yogurts (**CHAPTER VI**). These conditions were chosen due to the occurrence of the lactic acid fermentation at reasonable times to allow experiment execution and data generation. Here, the final yogurts were analyzed regarding their microbiological and physical properties, since pressure and temperature influence not only the metabolic activity of microorganisms, but also the physical properties of protein gel networks. Therefore, the viability of the starter cultures (*S. thermophilus* and *L. bulgaricus*) at the end of fermentation, whey separation and yogurt firmness were measured. Further characterization of the final yogurts was performed relying on a preliminary metabolomic approach (**CHAPTER VII**). In this case, only the yogurts produced at 10 MPa were analyzed, since fermentation presented the most interesting features when compared to the processes at atmospheric pressure. Metabolic abundance in each yogurt sample was profiled by ^{1}H NMR spectroscopy in order to study the influence of the fermentation conditions on the metabolome of the resultant yogurts, and possibly disclose some adaptation mechanisms from the starter cultures to withstand the non-conventional conditions used.

CHAPTER IV

Effect of decompression and compression procedures during fermentation process

4.1. Introduction

Fermentation under pressure is a novel application for high pressure (HP) technology (Aertsen et al., 2009; Mota et al., 2013). In this case, sub-lethal levels of pressure are used to cause a metabolic stress response by microorganisms (in order to adapt and survive at these conditions). As a consequence, this application can bring novel characteristics and features to both fermentative processes and final products (Mota et al., 2013). Few studies were already performed, applying this emergent HP approach to fermentative processes and changes in the fermentative rate and yield and/or shifts in the metabolic pathways with production of novel final products were observed (Bothun et al., 2004; Kato et al., 2007; Neto et al., 2016; Picard et al., 2007).

Our research group studied the application of HP to lactic acid fermentation for production of probiotic yogurt (Mota et al., 2015) and we detected a drawback in the process. Since the HP equipment used in this case had small dimensions (laboratory scale equipment with a pressure vessel with approximately 100 mL of capacity), it was only possible to pressurize one sample at a time. For example, when we performed a fermentation and was necessary to collect three samples during the fermentation time, we needed to perform three different pressure cycles: one for each sample. One way of overcoming this issue is to use a HP equipment with higher volume capacity and then more samples could be pressurized at a time. Therefore, the sample collection over time would be performed through the interruption of the pressurization cycle, saving experience time. In this case, the pressure vessel would be decompressed for a short time (only the time necessary to sample collection) and be compressed right after that. However, the effects of these decompression and compression procedures during the fermentative time were not evaluated yet and may influence the starter cultures fermentative capacity, having undesirable effects on the fermentative rate. Therefore, a preliminary study was performed to evaluate these possible effects on the fermentation under pressure, using yogurt production as a case-study. For that, fermentation at 10 MPa was conducted in a pilot-scale HP equipment with a pressure vessel of approximately 2 L capacity, where one fermentative process was performed with interruptions to collect samples during the time under pressure, and the other was performed without any interruptions.

4.2. Material and methods

4.2.1. Yogurt production

Milk preparation was performed based on Settachaimongkon et al. (2014) and Haque et al. (2001), with reconstitution of 10 % (w v^{-1}) Nido whole milk powder (Nestlé, Portugal) in distilled water to obtain a final liquid milk with approximately 9.7 % dry matter content. The prepared milk was pasteurized at 90 ºC for 20 minutes in a circulating water bath and it was then cooled rapidly to

ambient temperature by immersion in running tap water. Thereafter, milk was stored overnight at 5 ºC.

Sample preparation consisted in the combination of the pasteurized milk with a lactic acid culture (Yo-Aktiv of ADMIX Ltd. composed by *Lactobacillus bulgaricus* and *Streptococcus thermophilus*) at a concentration of 2 g L^{-1}, accordingly to the manufacturer's instructions. After homogenization, the mixture was transferred to a heat sealed plastic bag resistant to high pressures.

The mixture was then incubated at 43 ºC under pressure during 6 hours. The experiments were executed in a Hydrostatic press (FPG7100, Stanstead Fluid Power, Stanstead, United Kingdom) own by the Chemistry Department of University of Aveiro. The equipment has a pressure vessel of 100 mm inner diameter and 250 mm height surrounded by an external jacket to control the temperature. A mixture of propylene glycol and water was used as pressurizing fluid.

Fermentations were performed at 10 MPa, using fermentation under atmospheric pressure (0.1 MPa) as control. During fermentation time, several samples were collected and stored at -20 ºC. Each experiment and analysis was performed in duplicate.

4.2.2. Titratable acidity and pH

In this case, only acid production was monitored and determination of titratable acidity and pH variation were the physicochemical analyses used. Titratable acidity was analyzed using a Titromatic 1S (Crison Instruments, S. A., Spain), accordingly to Chandan and Kilara (2013) with some modifications: 1.50 mL of yogurt sample were diluted in 10.50 mL of water and then titrated with a 0.1N NaOH solution, until pH 8.9 was reached. The results obtained were expressed in % (w/w) of lactate. Additionally, pH of the fermentative medium was measured using a properly calibrated glass electrode (pH electrode 50 14, Crison Instruments, S. A., Spain), at 25 ºC.

4.3. Results and discussion

Fermentation at 10 MPa and 43 ºC (optimal temperature for lactic acid fermentation at 0.1 MPa) was used as a case-study to verify if the decompression and compression procedures during fermentation under pressure had effects on the fermentative rate, when compared to fermentation without these procedures. The results obtained are represented in Figure 4.1, corresponding to fermentations under pressure with and without interruptions for sample collection and using fermentation at 0.1 MPa as control. In this case, only the product formation over time was analyzed, i.e. pH variation and titratable acidity, expressed in lactate concentration.

In addition, this study also worked as a first test under pressure using milk powder and a lactic acid culture to perform the lactic acid fermentation. Since lactic acid fermentation performed by

Mota et al. (2015) used different milk and starter cultures, differences in the fermentative process and final product may have occurred, possibly reflecting in the fermentative rate.

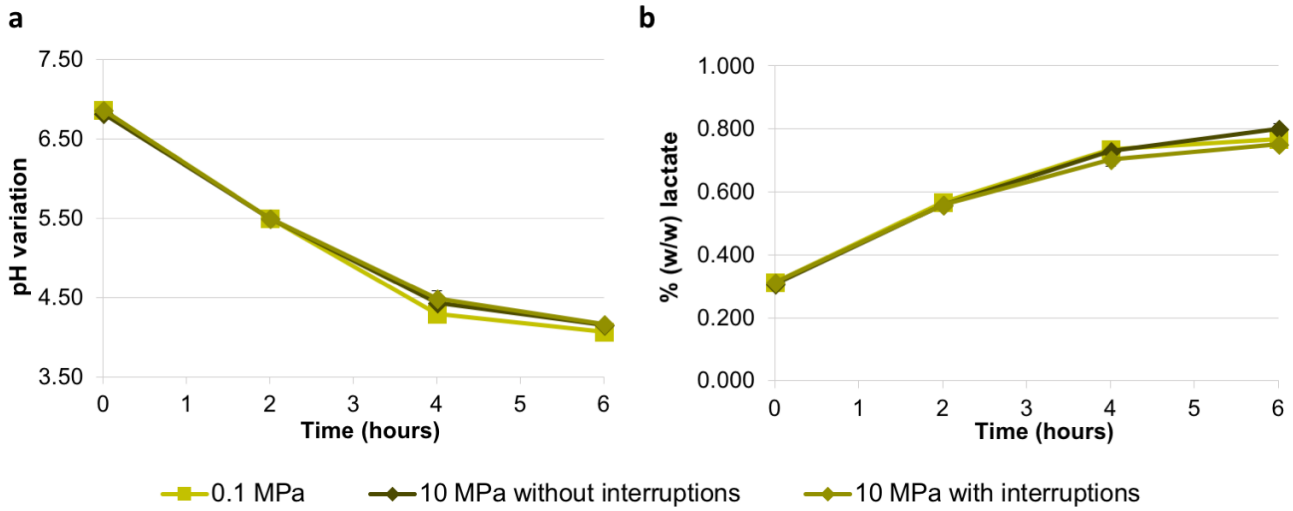

Figure 4.1. Variation of pH (a) and titratable acidity, expressed in lactate concentration, (b) over fermentation at 10 MPa and 43 ºC, with and without interruptions (diamonds) to collect samples during the fermentation time. Control fermentations at 0.1 MPa are represented as squares.

In general, both pH and titratable acidity are in accordance to each other, i.e. while pH decreased over time due to the increasing of acidity during the fermentative process, titratable acidity increased. Regarding the effect of decompression and compression during fermentation under pressure, similar profiles of pH variation and titratable acidity were observed in both fermentative processes. In fact, after 6 hours of fermentation at 10 MPa, a pH 4.15 and acidity of 0.752 % (w/w) were obtained for the fermentative process with interruptions to collect samples, while a pH 4.17 and acidity of 0.800 % (w/w) were obtained for the process without interruptions. Therefore, these two parameters show that the decompression and compression procedures do not affect the fermentative rate during fermentation under sub-lethal levels of pressure.

In addition, comparing the fermentation under pressure with the control, similar profiles were obtained in what regards to product formation over fermentation time. Taking into account that yogurt is obtained as a final product of lactic acid fermentation when a pH of 4.5 is reached (Hui et al., 2012), the time needed to produce yogurt is similar for fermentation at 10 and 0.1 MPa with yogurt produced after the 6 hours of fermentation. However, differences are easily identified when comparing these results with Mota et al. (2015), since the authors found that increasing pressure slowed down the fermentative rate. For instance, both pH variation rate and lactate production rate of fermentation at 5 MPa were lower than control fermentation, with lower pH values and lactate concentrations after 6 hours of fermentation. This difference can be explained by the different strains

of starter cultures used in this work, which can be more resistant to pressure than the ones used in the previous study, being able to overcome this stressful conditions and ferment at the same rate than at 0.1 MPa.

4.4. Conclusions

In sum, the decompression and compression procedures performed to collect samples during the fermentation under pressure did not present any effect on the fermentative process in what concern to the product formation rate. In fact, all the 3 fermentation processes evaluated in this chapter presented similar fermentative rates. Therefore, in the course of this work, the pilot-scale HP equipment with 2 L capacity was used and the sampling occurred during the time under pressure through decompression and compression of pressure vessel. In this way, experience time and experimental resources were saved, allowing to carry out the work within the fine frame of a Ph.D. thesis.

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CHAPTER V

Combined effect of pressure and temperature for yogurt production

This chapter is based on information of the following publication:
Lopes, R.P., Mota, M.J., Sousa, S., Gomes, A.M. Delgadillo, I., Saraiva, J.A., Combined effect of pressure and temperature for yogurt production. Submitted in Food Research International.

5.1. Introduction

High Pressure (HP) is a commercial processing technology usually applied for non-thermal pasteurization of foods. However, the pressure effect on microorganisms depends on the pressure magnitude, with increasing pressure leading to a progressive inactivation of proteins and cell damage (Abe, 2007). Recently, novel applications have been described for HP (Aertsen et al., 2009; Mota et al., 2013), some of them involving the use of sub-lethal levels of pressure as a mild stress condition to trigger general and specific stress responses by microorganisms (in a way to adapt and survive under these conditions). The activation of these stress responses usually involves changes in metabolic processes. This approach was also tested using some other emergent technologies (e.g., ultrasounds and electric fields). However, HP presented some advantages, namely in what regards to the possibility of applying the stress during the whole fermentation time without heating and the low energy requirements, reducing the process cost (Mota et al., 2018). Thus, the performance of fermentation under sub-lethal levels of pressure is an emergent concept, which may lead to changes in the fermentative rate and yield and/or shifts in the metabolic pathways with possible production of novel final products (Mota et al., 2013).

In fact, fermentative rate and yield of bioethanol production by *Saccharomyces cerevisiae* was enhanced by pressure levels of 5 and 10 MPa and the maximal ethanol production was obtained at 5 MPa (Picard et al., 2007). Regarding metabolic shifts under pressure, Bothun et al. (2004) observed a modification in product selectivity towards ethanol production rather than acetate by *Clostridium thermocellum* at 7.0 MPa and 17.3 MPa. In addition, bacterial cellulose produced by *Gluconacetobacter xylinus* under pressure was found to show profound morphological differences, when compared to the ones produced at atmospheric pressure (Kato et al., 2007). Another approach already tested is the application of HP-stresses only in the beginning of fermentation. Metabolic changes related to the production of lactate isomers by *Oenococcus oeni* were observed by Neto et al. (2016) when a stress of 100 MPa/8 h was applied. These differences suggest that sub-lethal levels of pressure may bring novel characteristics and features to both fermentative process and final product (Mota et al., 2013). Regarding food fermentations, HP was already applied during lactic acid fermentation for production of probiotic yogurt at 43 ºC, using commercial yogurt as inoculum (Mota et al., 2015). In this case, fermentative rate was found to decrease with increasing pressure until total inhibition at 100 MPa, but the extension of fermentation time at 5 MPa allowed the production of yogurt with the characteristic pH (pH 4.5).

In addition to pressure, temperature works as a thermodynamic variable, being widely used to investigate biological systems (Decaneto et al., 2015). Thus, the variation of fermentation temperature also has effects on fermentative processes and final products. For instance, in the case of yogurt production, the acidification rate and gel formation are highly affected by temperature (Lee and Lucey, 2004), with more viscous, smoother and slimy yogurts obtained when the temperature process is lowered from 43-45 ºC to 32-39ºC (Sodini et al., 2004).

Therefore, these two parameters (pressure and temperature) can be used together to modulate the fermentative processes, namely the metabolic activity of the microorganisms involved and possibly the characteristics of the final product. In fact, this approach was already applied to enzymatic systems, where acceleration of enzymatic reactions was obtained with the combination of pressure and temperature (Luong et al., 2016, 2015; Ueda et al., 1994; Van den Broeck et al., 2000), due to enzymes stabilization against thermal inactivation by pressure (Aertsen et al., 2009; Czeslik et al., 2017). However, this approach was never applied to fermentative processes, despite the possible entailed advantages. Therefore, the purpose of this work was to study the combined effect of pressure and temperature on fermentative processes, using yogurt production as a case-study. This dairy product was chosen because it corresponds to one of the most popular fermented product nowadays (Chilton et al., 2015) and yogurt production is a relatively fast process, facilitating the experimental process for data generation. Therefore, the fermentation process was performed under different combinations of pressure (10-100 MPa) and temperature (25-50 ºC), in order to understand the effects on the acidification rate of starter cultures present in yogurt.

5.2. Material and methods

5.2.1. Yogurt production

Milk preparation was performed based on Settachaimongkon et al. (2014) and Haque et al. (2001), with reconstitution of 10 % (w v^{-1}) Nido whole milk powder (Nestlé, Portugal) in distilled water to obtain a final liquid milk with approximately 9.7 % dry matter content. The prepared milk was pasteurized at 90 ºC for 20 minutes in a circulating water bath and it was then cooled rapidly to ambient temperature by immersion in running tap water. Thereafter, milk was stored overnight at 5 ºC.

Sample preparation consisted in the combination of the pasteurized milk with a commercial lactic acid lyophilized culture for yogurt production (Yo-Aktiv of ADMIX Ltd. composed by *Lactobacillus bulgaricus* and *Streptococcus thermophilus*) at a concentration of 2 g L^{-1}, accordingly to the manufacturer's instructions. After homogenization, the mixture was transferred to a heat sealed plastic bag resistant to high pressures.

The mixture was then incubated at different pressure and temperature conditions. The experiments were executed in two Hydrostatic presses (FPG13900 for room temperature experiments and FPG7100 for the remaining temperatures, both from Stanstead Fluid Power, Stanstead, United Kingdom) of our research group. While the FPG13900 equipment has three pressure vessels of 37 mm inner diameter and 520 mm height without temperature control, the FPG7100 equipment has a pressure vessel of 100 mm inner diameter and 250 mm height surrounded by an external jacket to

control the temperature. In both equipments, a mixture of propylene glycol and water was used as pressurizing fluid.

Pressures of 10, 30, 50 and 100 MPa and temperatures of ≈25 (room temperature, RT), 35, 43 and 50 ºC were tested, using fermentation under atmospheric pressure (0.1 MPa), and at the respective temperature, as control. During fermentation time, several samples were collected and stored at -20 ºC. Each experiment and analysis was performed in duplicate.

5.2.2. Physicochemical analyses

5.2.2.1.Titratable acidity and pH

Acid production was monitored by determination of titratable acidity and pH. Titratable acidity was analyzed using a Titromatic 1S (Crison Instruments, S. A., Spain), accordingly to Chandan and Kilara (2013) with some modifications: 1.50 g of yogurt sample were diluted in 10.50 mL of water and then titrated with a 0.1N NaOH solution, until pH 8.9 was reached. The results obtained were expressed in % (w/w) of lactate and the product formation rate r_P (mg g^{-1} h^{-1}) was calculated using the results correspondent to the exponential growth phase of the starter cultures.

Additionally, pH of the fermentative medium was measured using a properly calibrated glass electrode (pH electrode 50 14, Crison Instruments, S. A., Spain), at 25 ºC.

5.2.2.2.Sugar concentration

For determination of sugar concentration, yogurt samples were first treated with Carrez I and Carrez II solutions to precipitate proteins and other high molecular weight molecules, but keep carbohydrates in solution (Fisher et al., 2014). Initially, 1.00 g of yogurt samples was added to 60 mL of distilled water and the suspension was incubated at 50 ºC for 15 minutes. Then, 2 mL of Carrez I solution [potassium hexacyanoferrate (II) ($K_4[Fe(CN)_6].3H_2O$)], 2 mL of Carrez II solution [zinc sulphate ($ZnSO_4.7H_2O$)] and 4 mL of a 100 mM NaOH solution were added to the suspension. Finally, the mixture was diluted to a final volume of 100 mL with distilled water, mix thoroughly and the resulting solution was filtered through Whatman No. 1 filter paper (Megazyme, 2014).

Reducing sugars determination was measured according to the method described by Miller (1959), using 3,5-dinitrosalicylic acid (DNS) reagent. For that, 1.0 mL of the clear filtrate was added to 1.0 mL of DNS reagent and the mixture was incubated in a boiling water bath during 5 minutes. After cooling in an ice bath, the mixture was diluted with 10 mL of distilled water and the absorbance was measured at 540 nm. The concentration values were calculated using a calibration curve, obtained from glucose standard solutions, and were expressed in mg g^{-1} of yogurt. Additionally, the sugar consumption rate r_S (mg g^{-1} h^{-1}) was calculated taking into account only the results correspondent to the exponential growth phase of the starter cultures.

Regarding DNS reagent, it was prepared weighing 10 g of DNS and dissolving in 200 mL of a 2 N NaOH solution by heating with intensive stirring. Simultaneously, a solution of 300 g of potassium tartrate in 500 mL of distilled water was prepared by heating with intense stirring. Both solutions were mixed and stirred and the final mixture was then diluted to 1 L with distilled water.

5.2.3. Organic acids and sugars determination

Extraction of organic acids and sugars of yogurt samples was performed following the method described by da Costa et al. (2016), with modifications. Briefly, 1.00 g of yogurt was homogenized with 5 mL of 45 mmol L^{-1} for 1 min in a vortex and the mixture was then stirred in an orbital shaker for 30 min at 240 rpm. The homogenates were centrifuged at 6000 rpm for 30 min at 4 ºC and the supernatants filtered through a 0.22 μm pore size membrane filter and stored at -20 ºC until HPLC analysis. The chromatographic system consisted in a HPLC Knauer system equipped with a Knauer K-2301 RI detector, and an Aminex HPX-87H cation exchange column (300 x 7.8 mm) (Bio-Rad Laboratories Pty Ltd, Hercules, CA, USA). The mobile phase used was 13 mM H_2SO_4, delivered at a flow rate of 0.6 $mL.min^{-1}$ and the column maintained at 65 °C. Peaks were identified by their retention times and quantified using calibration curves prepared with different standards.

With the results obtained, some fermentation kinetic parameters were calculated based on da Fonseca (2007), including percentage of substrate consumption (% (w/w)), productivity Q_P (mg g^{-1} h^{-1}), product yield on substrate $Y_{P/S}$ (g_P g_S^{-1}) and fermentation efficiency (% (w/w)).

5.2.4. Statistical analysis

The results obtained were tested at a 0.05 probability level and the combined effect of pressure and temperature was tested with a one-way analysis of variance (ANOVA), followed by a multiple comparisons test (Tukey HSD) to identify statistical significant differences between samples.

5.3. Results and discussion

5.3.1. Effect of pressure and temperature on yogurt fermentation

Lactic acid fermentation was performed under several combinations of pressure and temperature. Initially, the effect of increasing pressure was only studied at 43 ºC, the optimal temperature of yogurt fermentation at atmospheric pressure (used in industry). Then, in order to observe the temperature influence on fermentation under pressure, a large spectrum of temperatures that can be used without inhibit the fermentation was tested (ranging from room temperature at ≈25 ºC (RT) to 50 ºC). Fermentation was monitored by pH variation (Figure 5.1), which is one of the most important physicochemical parameters in yogurt production, since the yogurt production process is considered finished when a pH of 4.5 is reached (corresponding to the isoelectric point for casein) (Hui et al.,

2012). Thus, this value is represented by a dotted line in Figure 5.1, to easily identify the time needed for yogurt production in all cases studied.

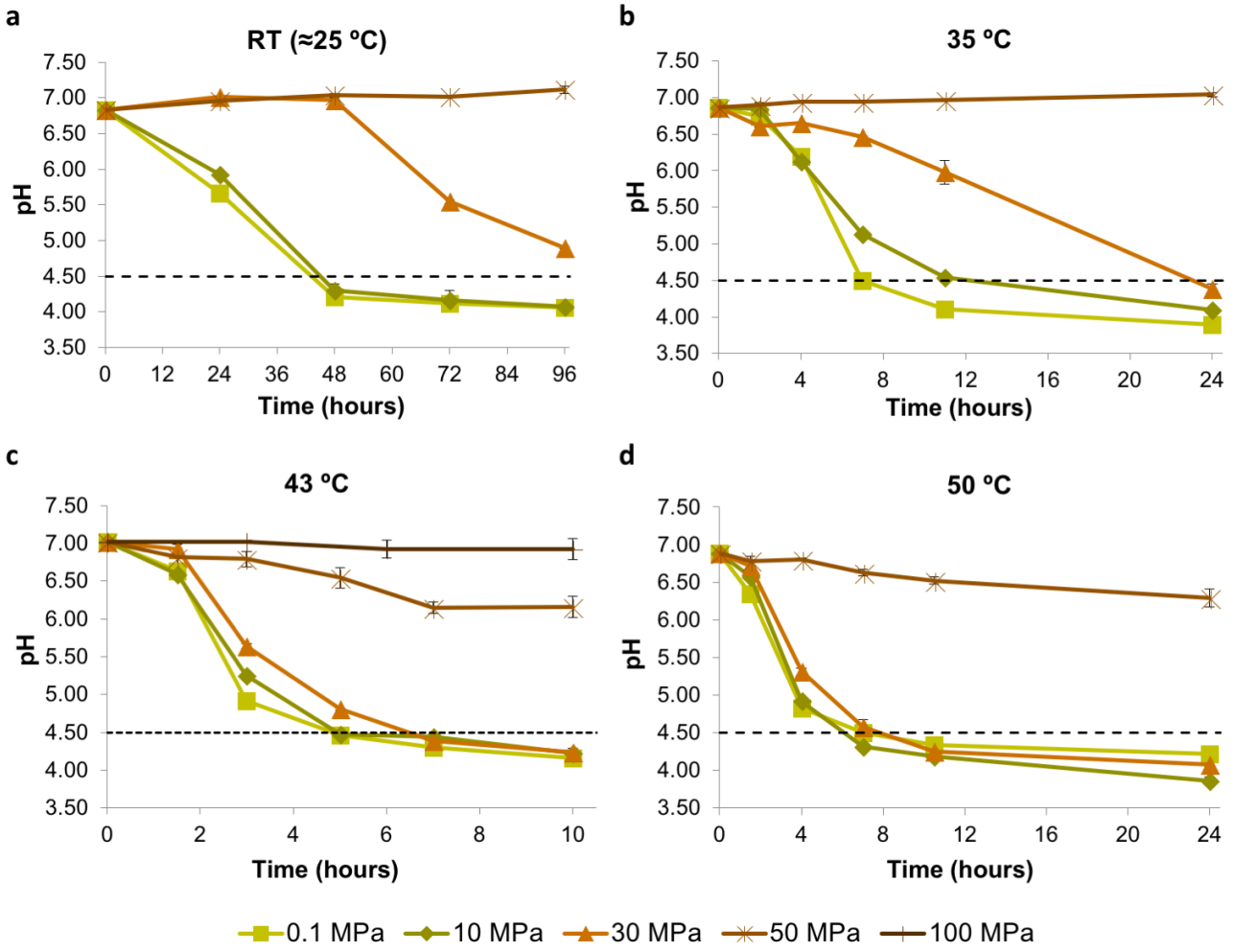

Figure 5.1. pH variation during fermentation at room temperature (≈25 ºC) (a), 35 ºC (b), 43 ºC (c) and 50 ºC (d), under different conditions of pressure: 10 MPa (diamonds), 30 MPa (triangles), 50 MPa (stars) and 100 MPa (crosses). Control fermentations at 0.1 MPa are represented as squares.

In general, differences in the fermentative profiles were observed for each combination of pressure and temperature. Fermentation at atmospheric pressure was influenced by the temperature changes, decreasing the fermentative rate at RT, 35 and 50 ºC relatively to 43 ºC, and consequently increasing the time required to obtain yogurt. Thus, fermentation times were adapted for each set of temperature experiments (10 h for 43 ºC experiments; 24 h for 35 and 50 ºC experiments and 96 h for RT experiments). Lee and Lucey (2003) and Nguyen et al. (2014) also observed this decrease in the fermentative rate when an incubation temperature different from the optimal was used for yogurt production at atmospheric pressure. While slower enzymatic reactions and membrane solidification are behind the lower microbial growth rate when temperature decreases, structural cell components

denaturation and enzyme inactivation are behind it when temperature is higher than optimal (FDA, 2003).

Regarding the pressure influence, the increase of pressure was generally reflected in the decrease of pH variation rate. However, this effect was also dependent on the temperature applied in each case. For instance, fermentation at 10 MPa and 0.1 MPa showed similar profiles in almost all temperatures, except for 35 ºC, where the fermentative rate was slightly lower with a final pH slightly higher ($p < 0.05$) than 0.1 MPa. Increasing the pressure to 30 MPa slowed down fermentation at all temperatures, more considerably in some cases than in others. While at 43 and 50 ºC only a slight decrease was observed (with similar final pHs obtained at 43 ºC ($p > 0.05$), and close final pHs at 50 ºC, but significantly different ($p < 0.05$)), a substantial decrease was observed at 35 ºC and RT ($p < 0.05$). In fact, yogurt typical pH was obtained for all fermentations at 10 and 30 MPa, with the exception to 30MPa/RT, within the longest fermentation time studied (96 h). However, the farther the fermentation conditions were from the conventional one (i.e., 0.1 MPa/43 ºC), the longer the fermentation times needed to produce yogurt. On the other hand, no substantial fermentation occurred when pressure increased to 50 and 100 MPa, with no pH variation at 50 MPa/RT, 50 MPa/35ºC and 100 MPa/43 ºC.

In summary, an increase of the pressure inhibitory effect was observed when temperature decreased, which was emphasized by the different pressures levels needed to inhibit fermentation at each temperature tested: 50 MPa at RT and 35 ºC, in contrast to 100 MPa at 43 ºC. Pressure increasing leads to inhibition of some cell processes and metabolic reactions essential for cell maintenance, depending on pressure resistance of the cell structure (Mota et al., 2018). For instance, cell membrane is one of the most pressure sensitive cellular components, among biological systems. As occurring at low temperatures, membrane fluidity decreases with pressure increasing, reducing the membrane permeability and consequently disrupting cell metabolism (Winter and Jeworrek, 2009). Thus, the combination of high pressures and low temperatures seems to compromise both cell structure and function to a higher extent than each non-optimal condition separately, since both have negative effects on cells.

On the other hand, microorganisms can withstand pressure due to the production of proteins that are able to protect cells against heat and pressure treatments (Abee and Wouters, 1999; Welch et al., 1993). Thus, cells at high pressures and high temperatures are able to withstand these severe conditions more easily than cells at high pressures and low temperatures. In fact, higher temperatures cause an increment of membrane permeability (Chandler, 2017; Winter and Jeworrek, 2009), which compensates the opposite effect of high pressure effect on membranes. This can explain the fact that the increase of the inhibitory effect of pressure was not observed at 50 ºC, even though fermentation was longer than at 43 ºC. In fact, the fermentation profiles at 10 and 30 MPa were similar to the control fermentation at 50 ºC.

Comparing the results obtained at 43 ºC with Mota et al. (2015), fermentative rates also decrease with pressure increase, until no fermentation occur at 100 MPa. However, fermentation at 5 MPa already presented a decrease in the pH variation rate, in contrast to the present work, where fermentation at 0.1, 10 and 30 MPa presented similar profiles. These differences may be explained by the use of a different inoculum for yogurt production with different pressure resistance. In fact, commercial lyophilized starter cultures were used as inoculum in the present work, while commercial yogurt was used in Mota et al (2015).

5.3.2. Product formation and substrate consumption rates

In addition to pH variation, titratable acidity and reducing sugars concentration were also monitored to give information about the product formation and substrate consumption, respectively. The results obtained for titratable acidity and reducing sugars concentration are shown in Figure A.1 and A.2 (Appendix A), respectively. With these results, the respective rates were calculated using the results correspondent to the exponential growth phase of the starter cultures.

Both fermentative rates (Figure 5.2) are in accordance to the pH variation results: i) increased as temperature increase up to 43 ºC, corresponding to an acceleration of fermentation; and ii) decreased, even if slightly, as temperature increase to 50 ºC, corresponding to a deceleration. Thus, the fastest yogurt production occurred at 43 ºC for all pressures tested (ranging from atmospheric pressure (0.1 MPa) to 50 MPa), which is usually reported as the optimal temperature for yogurt production at atmospheric pressure.

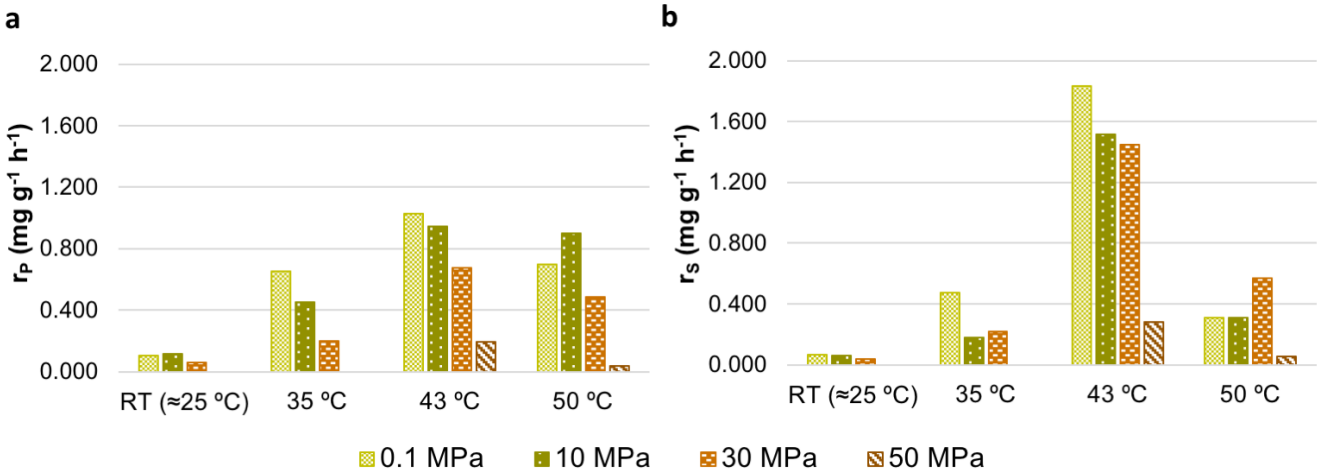

Figure 5.2. Product formation rate (a) and substrate consumption rate (b) correspondent to fermentation at room temperature (≈25 ºC), 35 ºC, 43 ºC and 50 ºC, under different conditions of pressure: 0.1 MPa, 10 MPa, 30 MPa and 50 MPa.

Regarding the pressure effect, fermentative rates mostly decreased with pressure increasing, with exception of fermentation at 50 ºC where the product formation rate (r_P, Figure 5.2a) was higher at

10 MPa and the substrate consumption rate (r_S, Figure 5.2b) was higher at 30 MPa. In this case, the higher r_P at 10 MPa may indicate that low pressures accelerate the lactic acid fermentation at 50 ºC, which do not occur at lower temperatures. A similar rate enhancement was already reported by Picard et al. (2007) for alcoholic fermentation by *S. cerevisiae* that was accelerated when fermentation occurred under pressure (5 and 10 MPa) with production of higher amounts of bioethanol. In fact, higher titratable acidities, expressed as % (w/w) lactate, were also achieved when lactic acid fermentation occurred at 10 MPa/50 ºC (Figure A.1 in Appendix A). However, regarding r_S, the acceleration of substrate consumption at 10 MPa/50 ºC was not detected, with r_S similar to the one at 0.1 MPa. But, the highest r_S was obtained for the fermentation at 50ºC/30 MPa, which may be related with the need of more energy, i.e. more substrate, to withstand these harsh levels of pressure and temperature, in order to microbial cells survive, ferment and produce yogurt.

In summary, antagonistic effects on fermentation seem to occur when both pressure and temperature increase up to 43 ºC, since fermentation is accelerated by the temperature increase on one hand, and, on the other hand, is slowed down by the pressure increase. However, when temperature was increased to 50 ºC, this antagonistic effect was basically not verified, since fermentation was slowed down by temperature (and not accelerated, as expected) and, on the other hand, pressure did not slow down fermentation. Different stress responses could be behind this different behavior of cells towards pressure. As stated in the previous section, the production of heat-shock proteins caused by pressure increasing may help to withstand this higher temperature. In fact, the biosynthesis of proteins involved in the prevention of thermal degradation is already documented as one of the mechanisms of stress resistance to pressure for some lactobacilli strains (Bucka-Kolendo and Sokołowska, 2017).

5.3.3. Organic acids and sugars assessment

In order to deepen the study about the effect of pressure and temperature on yogurt fermentation, the presence of organic acids and sugars in the extracellular medium was evaluated by HPLC analysis. In this case, only fermentations at 10 and 30 MPa were studied, with fermentation at 0.1 MPa used as control. These cases were selected because yogurt was obtained within an experimental reasonable fermentation time, thus facilitating the experiments execution and data generation. The only exception was fermentation at 30 MPa/RT, where yogurt was not produced at the end of fermentation time but fermentation was almost complete.

Lactose, galactose, glucose, lactate and citrate were identified in all samples analyzed and their variation throughout fermentation is represented in Figure 5.3. However, since the concentration of citrate remained approximately constant over the fermentation time, these results were not included in Figure 5.3. The presence of citrate in the samples is explained by its presence on the milk used in this work. In fact, citrate is the predominant organic acid in milk (Costa et al., 2015). Regarding the other compounds identified, lactose concentration had the tendency to decrease over fermentation

time, while galactose and lactate increased and glucose remained constant during almost all fermentations tested.

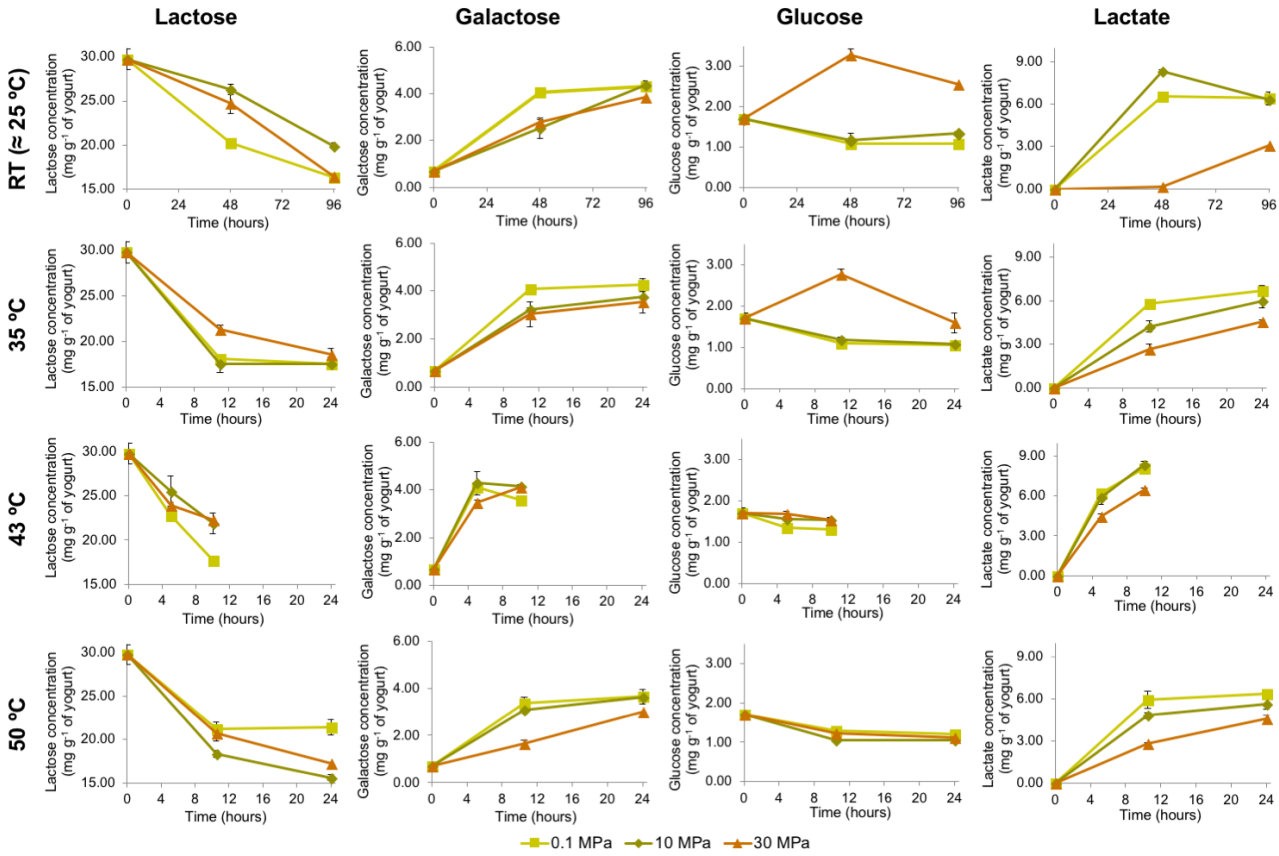

Figure 5.3. Lactose, galactose, glucose and lactate concentrations during fermentation at room temperature (≈25 ºC), 35 ºC, 43 ºC and 50 ºC, under different conditions of pressure: 10 MPa (diamonds) and 30 MPa (triangles). Control fermentations at 0.1 MPa are represented as squares.

Lactose is the major component of milk (with a concentration of 29.77 mg g^{-1} in the present work) and is the main substrate used by lactic acid bacteria during fermentation. Thus, as expected, lactose concentration decreased over time in all cases studied. However, different profiles were observed for each fermentation analyzed. While lactose concentration showed a linear decreasing pattern during fermentations at RT and 43 ºC, a marked decrease followed by a stabilization was observed at 35 and 50 ºC. In addition, pressure also affected lactose consumption, which was reflected in the different final concentrations obtained in each case. In general, higher final concentrations were achieved when fermentation occurred at higher pressures ($p < 0.05$), i.e. when fermentation was slower, indicating that lactose consumption was lower in these cases. However, some exceptions were observed. For instance, at RT, fermentation at 10 MPa presented a higher final concentration than fermentation at 0.1 and 30 MPa, which had similar final values ($p > 0.05$). This unexpected higher lactose consumption at 30 MPa may be related with the need of energy by the cells to trigger

adaption mechanisms to the harsh conditions they were subjected to. In fact, while pH variation did not occur in the first 48 hours, approximately 20 % of lactose was already consumed in this case. The other exception was observed at 50 ºC, where the fermentation at 0.1 MPa presented a higher final concentration than fermentation at 10 and 30 MPa. In this case, the final pH obtained was also slightly higher at 0.1 MPa than 10 and 30 MPa, which may explain this difference in lactose consumption.

In addition to lactose, galactose and glucose were also present in the samples. During fermentation, lactose is transported into the cell by permeases without any chemical modification, being afterwards hydrolyzed by β-galactosidase to glucose and galactose. Usually, glucose is catabolized via Embden–Meyerhof–Parnas (EMP) pathway, being galactose secreted from the cell (Tamime and Robinson, 1999). Thus, variation of galactose concentration during fermentation must be related with lactose variation, i.e. galactose concentration should increase when lactose concentration decreased. In fact, galactose concentration increased over time in all fermentations tested, with different variation profiles, as observed for lactose. In general, galactose concentration had a marked increase in the beginning of fermentation followed by a slight stabilization. The exceptions to this profile occurred when fermentation was slower (e.g., 10 MPa/RT, 30 MPa/RT, 30 MPa/50 ºC) with concentration increasing during all the fermentation time. Comparing with lactose variation, some differences were observed: i) at 35 ºC, the variation of lactose concentration at 30 MPa was slower than at the other pressures tested, while galactose variation was similar to 10 MPa; ii) at 43 ºC, lactose was more consumed at 0.1 MPa, resulting in a lower final concentration ($p < 0.05$), but the final concentrations of galactose was similar for all pressures tested ($p > 0.05$); and iii) at 50 ºC, while lactose consumption was lower during the fermentation at 0.1 MPa, a lower increase of galactose concentration was observed at 30 MPa, resulting in lower final concentrations ($p < 0.05$). Therefore, these differences may indicate changes in lactose metabolism, due to the combined effect of pressure and temperature.

In contrast to galactose, glucose is catabolized to pyruvate right after lactose hydrolysis and not expelled to the extracellular medium (Tamime and Robinson, 1999). Thus, it would be expected that glucose concentration in the extracellular medium would remain constant or even decrease during fermentation time. In fact, this behavior was observed in almost all fermentations tested, with the exception of fermentation at 30 MPa/RT and 30 MPa/35 ºC. In these cases, an increase of glucose content was observed in the beginning of fermentation, being followed by a decrease. Interestingly, these two conditions correspond to the fermentations with a lower fermentative rate (r_P, Figure 5.2a). One possible explanation may be that lactose hydrolysis and glucose catabolism are not affected by pressure to the same extent, resulting in an excess of glucose produced by lactose hydrolysis, when compared to the amount used to proceed the fermentative process. Thus, cells might expel this excess, increasing the glucose concentration in the extracellular medium. When the fermentation rate increased, more glucose is consumed, less is expelled and its concentration in the medium decreased.

In fact, Neto et al. (2016) verified that enzymes can be more resistant to pressure than the microbial cell where they are present, since although pressure caused complete inactivation of *O. oeni*, malolactic enzyme maintained some residual activity. Therefore, pressure may have a similar effect on β-galactosidase and starter cultures of yogurt – *S. thermophilus* and *L. bulgaricus*.

With the results obtained for sugar content, the percentage of substrate consumption was calculated (Figure 5.4a). Taking into account that both galactose and glucose may be metabolized by the starter cultures (Hardie, 1986; Kandler and Weiss, 1986), substrate consumption was determined by mass balances. In most cases, about 30 % of the sugars present in milk were consumed by the starter cultures, with slight lower values obtained at higher pressures. However, some exceptions were observed: lower values were observed at 10 MPa/RT, 10MPa/43 ºC, 30 MPa/43 ºC and 0.1 MPa/50 ºC, and, on the other hand, a slightly higher value was observed for fermentation at 10 MPa/50 ºC. Interestingly, these differences correspond to lower and higher variations of lactose concentration, respectively. Thus, lactose variation is the predominant parameter in respect to substrate consumption during yogurt production.

Regarding acids produced during fermentation, lactate is the main product of carbohydrate metabolism of lactic acid fermentation. Yogurt bacteria usually perform homolactic fermentation, where only lactate is produced from pyruvate (Tamime and Robinson, 1999). Thus, lactate must be the main acid responsible for the acidity increase in yogurt samples. Analyzing the obtained results, lactate production was found to vary accordingly to the pH variation (Figure 5.1). For instance, the production was inhibited by increasing pressure at all temperatures tested, with similar lactate concentrations obtained at the end of fermentations at 0.1 and 10 MPa (statically similar values were obtained at RT and 43 ºC ($p > 0.05$) and close but significantly different values were obtained at 35 and 50 ºC ($p < 0.05$)), but lower concentrations at 30 MPa ($p < 0.05$). In fact, during fermentation at 30 MPa/RT, lactate production only occurred between 48 and 96 hours of fermentation, due to the lowest fermentative rate observed. Interestingly, this effect was not observed in lactose concentration that decreased during the whole fermentation time, in contrast to glucose that reached the highest concentration after the 48 hours of fermentation. Therefore, these results support the explanation given above, suggesting that lactose hydrolysis and lactate production were not affected by pressure to the same extent.

Pressure inhibition of lactate production was reflected by lower productivities (Q_P) at 30 MPa for each temperature tested (Figure 5.4b). Fermentations at 43 ºC presented higher Q_P values, which was expected since lower fermentative times were needed to obtain yogurt at 43 ºC. Thus, similar values were obtained at 35 and 50 ºC (24 hours of fermentation in both cases) and lower ones at RT (96 hours of fermentation).

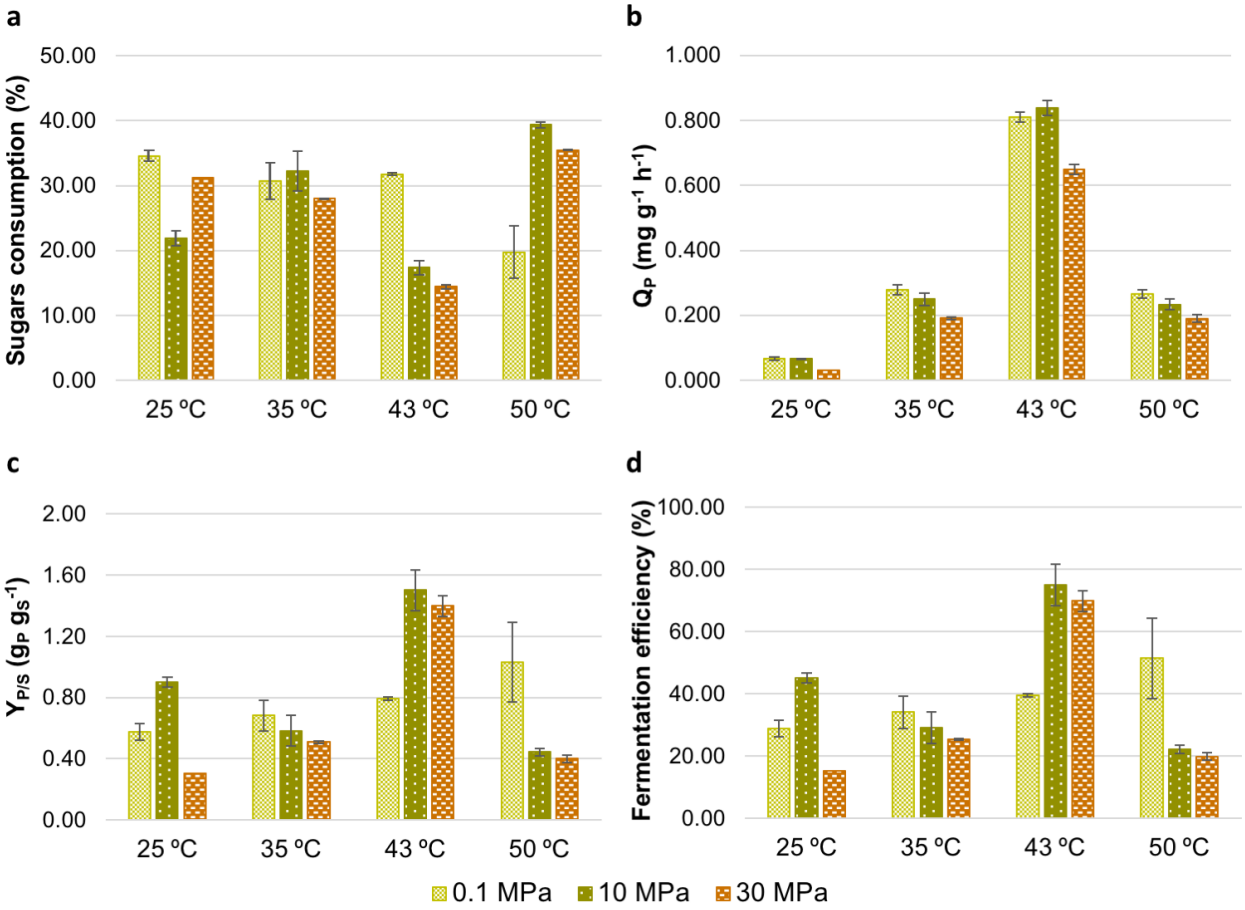

Figure 5.4. Consumed sugars (a), lactate productivity (b), lactate on sugars yield (c) and lactate efficiency (d) correspondent to fermentation at room temperature (≈25 ºC), 35 ºC, 43 ºC and 50 ºC, under different conditions of pressure: 0.1 MPa, 10 MPa and 30 MPa.

In order to relate lactate production to the sugar consumption, two kinetic parameters were calculated – fermentation yield and efficiency (Figures 5.4c and 5.4d, respectively). While fermentation yield gives information about the amount of lactate produced per sugar consumed, fermentation efficiency is the percentage of lactate that was actually produced relatively to the amount that could be theoretically produced with the sugars consumed during the process (da Fonseca, 2007). Analyzing the results obtained for both parameters, similar profiles were observed, i.e. higher yields correspond to higher efficiencies. However, a standard profile for pressure influence was not clearly identified. Generally, pressure increasing seemed to decrease fermentation yield and efficiency, i.e. sugars were consumed but lactate was not produced to the same extent under pressure, which may suggest that sugars were used by bacteria to other cellular processes (such as, adaptation mechanisms to pressure), but not for lactate production. However, some exceptions were observed at 10 MPa/RT, 10 MPa/43 ºC and 30 MPa/43 ºC, which presented higher values of yield and efficiency than the respective control samples (at atmospheric pressure). In these cases, bacteria were

able to produce high concentrations of lactate with less sugars consumed, indicating that sugar catabolism towards lactate production was improved at these conditions.

Interestingly, analyzing the temperature influence at each pressure, different profiles were observed when fermentation was performed under pressure, compared to atmospheric pressure. While yield and efficiency increased with temperature increasing up to 50 ºC at 0.1 MPa, under pressure, higher values were observed at 43 ºC. In fact, values higher than 0.1 MPa were observed under pressure, with fermentation efficiencies of 75.09 % and 69.89 % at 10 and 30 MPa, respectively, against 39.63 % at 0.1 MPa. Improved fermentative yields at 10 MPa were also reported by Picard et al. (2007) during alcoholic fermentation. The authors assumed that this increased activity under pressure might be related with the enhancement of glucose uptake, glycolysis and/or fermentation pathways, which can also explain the results obtained here.

Therefore, this work provided the first results about the combined effect of pressure and temperature on microbial fermentation, applied to yogurt production. All the results presented in this work pointed that the most suitable conditions for yogurt fermentation were, in fact, at 43 ºC, where lower fermentation times were required to produce yogurt and higher lactate productivities were achieved. However, the optimal conditions observed were 10 MPa/43 ºC, being even better than fermentation at 0.1 MPa. Thus, fermentation under sub-lethal levels of pressure can bring relevant improvements to the fermentative process, namely lower sugars consumption, higher productivity, yield and efficiency, when compared to fermentation at atmospheric pressure. These changes may indicate changes in the metabolic activity of microorganisms under pressure, with the metabolic pathway of lactate production being stimulated, while other pathways were reduced, increasing lactate productivity, yield and efficiency, as a consequence.

5.4. Conclusions

This work gives the first insights on the combined effect of pressure and temperature on a microbial fermentation process and kinetics. Simultaneous variation of both pressure and temperature influenced the fermentative rates, with the pressure effect being dependent on incubation temperature. In general, higher pressures and lower temperatures slowed down yogurt production, with fermentations at 43 ºC presenting the highest fermentative rates. Using kinetic parameters to characterize the influence of both variables on the fermentative process, interesting differences in the processes fermented under pressure were achieved. Improved yields were observed for fermentations under pressure (10 and 30 MPa) at 43 ºC, which were reflected into lactate efficiencies of 70-75 %, in contrast to 40 % at atmospheric pressure. Thus, the fermentative process showed modifications under pressure, with microorganisms more effectively converting lactose into lactate. Therefore, pressure and temperature may be used as process variables to modulate the metabolic activity of microorganisms during fermentation and improve the productivities and yields of the desired

product. Since these modifications may be converted in a final product with different properties, the yogurt produced under pressure should be analyzed regarding its microbiological, rheological, sensorial and nutritional properties, in order to describe the pressure influence on the final product of fermentation.

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CHAPTER VI

Physicochemical and microbial changes in yogurts produced under different conditions of pressure and temperature

This chapter is based on information of the following publication:

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6.1. Introduction

Yogurt is, by definition, a coagulated milk product, resulting from the milk fermentation by starter cultures, composed by *Streptococcus thermophilus* and *Lactobacillus bulgaricus* (Adolfsson et al., 2004; Belitz et al., 2009). The acidification process causes caseins' aggregation around pH 4.7 (isoelectric point), which results in a final product with a three-dimensional network consisting of casein micelles surrounded by fat and serum globules, gel-like texture and characteristic taste (due to lactate production) (Haque et al., 2001; Mulvihill et al., 1995). Thus, lactic acid fermentation induces significant structural changes, which are responsible for the final physical properties of yogurt (such as texture, stability and consistency) (Gastaldi et al., 1997; Lucey and Singh, 1997). These physical properties are among the main parameters for evaluation of yogurt quality and play an important role in consumer acceptance (Lee and Lucey, 2004).

Incubation temperature during the production process has major effects on the physical properties of final yogurt due to the significant impact on gel formation and acidification rate (Laligant et al., 2003; Lee and Lucey, 2003; Purwandari et al., 2007; Sodini et al., 2004; Tamime and Robinson, 1999; Wu et al., 2009). For instance, despite being more advantageous in the industrial process, higher incubation temperatures lead to several defects on final yogurt, such as increase of whey separation (Lee and Lucey, 2004, 2003; Purwandari et al., 2007), a weaker protein network with a coarser microstructure (Lee and Lucey, 2004; Lucey and Singh, 1997) and, consequently, a decrease in gel firmness, viscosity and smoothness, and decrease in desirable sensory properties (Tamime and Robinson, 1999; Wu et al., 2009). On the other hand, incubation at a lower temperature range is associated with higher production time and costs, but also to an improvement of several sensorial properties, with yogurts being more viscous, smoother and slimy when temperature is reduced from 43-45 ºC to 32-39 ºC, due to the denser protein matrix obtained under these conditions (Hammelehle et al., 1998; Lucey et al., 1998b; Nguyen et al., 2014; Sodini et al., 2004).

Another thermodynamic parameter that induces changes on the physical properties of protein gel networks is pressure, since it causes modifications on protein aggregation/disaggregation due to the weakening of electrostatic and hydrophobic interactions (Funtenberger et al., 1997; Heremans and Smeller, 1998). Therefore, the high pressure (HP) technology, which is commonly used for food pasteurization (Huang et al., 2014), may also be used for the modification of the physical structure of food biopolymers (such as proteins) (Correia et al., 2011; Ferrão-Gonzales et al., 2000; Foguel et al., 2003; Knorr et al., 2006), producing food products with improved texture properties and water holding capacity (Anema, 2010; Cadesky et al., 2017; Kaur et al., 2013; Sikes et al., 2009; Yang et al., 2015).

In addition, the performance of microbial fermentations under pressure is an emergent application of HP technology. In this case, sub-lethal levels of pressure (5-50 MPa) are used to trigger specific stress responses by microorganisms in order to obtain pressure-adapted cells, able to survive at these conditions. Thus, changes in the metabolic processes occur and the fermentative processes and/or

the final products may present different characteristics when compared to the products obtained by conventional processes (Mota et al., 2018, 2013). Changes in the fermentative rate and yield (Picard et al., 2007), metabolic shifts in the product selectivity (Bothun et al., 2004), and production of polymers with profound morphological differences (Kato et al., 2007), were already reported for fermentative processes that took place under pressure. Taking this into account, sub-lethal levels of pressure were applied to yogurt production by Mota et al. (2015), where fermentative rate decreased with increasing pressure up to 100 MPa. In spite of that, observed improvements of yield and lactate efficiency at 10 MPa and 43 ºC were observed in Chapter V, while studying the effect of the combination of pressure (10-100 MPa) and temperature (25-50 ºC) on lactic acid fermentation.

Overall, pressure and temperature influence not only the metabolic activity of microorganisms, but also the physical properties of protein gel networks. Thus, the purpose of this work was to study the effect of both pressure and temperature on yogurt production. For such, the fermentation process was performed under different combinations of pressure (10 and 30 MPa) and temperature (35, 43 and 50 ºC), and the produced yogurts were analyzed regarding their microbiological and physical properties, i.e. the viability of the starter cultures at the end of fermentation, whey separation and yogurt texture.

6.2. Material and methods

6.2.1. Yogurt production

Milk preparation was performed based on Settachaimongkon et al. (2014) and Haque et al. (2001), by reconstitution of 10 % (w v^{-1}) Nido whole milk powder (Nestlé, Portugal) in distilled water to obtain a final liquid milk with approximately 9.7 % dry matter content. The prepared milk was pasteurized at 90 ºC for 20 minutes in a circulating water bath and it was then cooled quickly to room temperature by immersion in running tap water. Thereafter, milk was stored overnight at 5 ºC.

Sample preparation consisted in the combination of the pasteurized milk with a lactic acid culture (Yo-Aktiv of ADMIX Ltd., Bulgaria, composed by *L. bulgaricus* and *S. thermophilus*) at a concentration of 2 g L^{-1}, according to the manufacturer's instructions. After homogenization, the mixture was transferred to heat sealed polyamide–polyethylene bags (PA/PE-90, Plásticos Macar – Indústria de plásticos Lda., Portugal) in the case of chemical and microbiological analyses and syneresis, and to plastic polyethylene–terephthalate containers (Espaçoplás Indústria e Comercialização de Plásticos Lda, Portugal) of 125 mL (to have enough amounts of yogurt to carry out the textural analysis). All the bags and containers were sterilized by UV-light (for 15 min) in a laminar flow cabinet (BioSafety Cabinet Telstar Bio II Advance, Spain) before being filled with the mixture.

The mixture was then incubated at different pressure and temperature conditions. The experiments were done in a high pressure equipment (FPG7100, Stanstead Fluid Power, United Kingdom). The equipment has a pressure vessel of 100 mm inner diameter and 250 mm height surrounded by an external jacket to control the temperature. A mixture of propylene glycol and water (40:60 v/v) was used as pressurization fluid.

Fermentation under three pressure levels (10 and 30 MPa) and temperatures (35, 43 and 50 ºC) took place, using fermentation under atmospheric pressure (0.1 MPa) for each temperature abovementioned, as control. Fermentation times were selected according to the observed fermentative rates at each temperature: 24 hours at 35 and 50 ºC, and 10 hours at 43 ºC. The final product of each fermentation was collected and stored at -20 ºC. Each experiment and analysis was performed in duplicate, with exception of textural analysis where samples in quadruplicate were analyzed.

6.2.2. Chemical analysis

The pH of the final yogurt was measured using a properly calibrated glass electrode (pH electrode 50 14, Crison Instruments, S. A., Spain), at 25 ºC. Lactate production and lactose consumption were determined by HPLC analysis. Firstly, extraction of organic acids and sugars was performed following the method described by da Costa et al. (2016), with minor modifications. Briefly, 1.00 g of yogurt was homogenized with 3 mL of 45 mmol L^{-1} H_2SO_4 for 1 min in a vortex and the mixture was then stirred in an orbital shaker for 30 min at 240 rpm. The homogenates were centrifuged at 6000 rpm for 30 min at 4 ºC and the supernatant was filtered through a 0.22 μm pore size membrane filter and stored at -20 ºC until HPLC analysis. The chromatographic system consisted in a HPLC Knauer system equipped with Knauer K-2301 RI detector and an Aminex HPX-87H cation exchange column (300 x 7.8 mm) (Bio-Rad Laboratories Pty Ltd, Hercules, CA, USA). The mobile phase was 13 mmol L^{-1} H_2SO_4, delivered at a flow rate of 0.6 mL min^{-1} and the column was maintained at 65 °C. Peaks were identified by their retention times and quantified using calibration curves prepared with appropriate standards.

6.2.3. Microbiological analysis

Lactic acid bacteria in yogurt were determined according to the method described by Rotar et al. (2015). For that, yogurt samples were ten-fold diluted in Ringer's solution, homogenized and prepared in duplicate. The viable counts of *S. thermophilus* were determined using M17 agar (Oxoid, England) and aerobic incubation at 37 ºC for 24 h. The viable counts of *L. bulgaricus* were determined using double-layer agar plates of *de* Man, Rogosa, and Sharpe agar (MRS; Merck, Germany) and aerobic incubation at 37 ºC for 72 h. Plates containing 15 to 300 colony-forming units (CFU) g^{-1} were counted, and the counts were expressed as $\log_{10}$ CFU g^{-1} of yogurt.

6.2.4. Syneresis

Amount of syneresis was measured according to the method described by Karnopp et al. (2017). Ten grams of yogurt samples were centrifuged at 7,870 g for 10 min at 4 ºC. Then, the clear supernatant was poured off, weighed and the percentage of syneresis was calculated according to the following equation:

$$Syneresis\ (\%) = \frac{expelled\ whey\ (g)}{yogurt\ mass\ (g)} \times 100$$

6.2.5. Textural analysis

The texture of the yogurts was accessed using an adapted penetration probe (Perspex cylindrical probe with 25 mm diameter and 35 mm length) fitted to a TA.HDi texture analyzer (Stable Micro Systems, England) equipped with a 5 kg load cell. Four yogurt samples in 125 mL plastic bottle containers with 45x45x60 mm of width x depth x height, (filled with 120 mL of the mixture milk/cultures and fermented as described above) were tested by uniaxial penetration measurements. Samples were kept at 4 °C until the measurement at room temperature. Penetration tests were carried out to a depth of 30 mm at a 0.5 mm s^{-1} rate. The Stable Micro Systems' Texture Expert Exceed software was used to extract some mechanical parameters from the force *vs.* distance curves: firmness (N), firmness work done (mJ) and adhesion force (N). Firmness was defined as the maximum force required to achieve a given deformation, being the peak force of the penetration cycle. Firmness work done was defined as the energy required to drive the probe during the downward penetration step, being the area under the positive peak. Adhesive force was defined as the maximum force generated during the probe upstroke, being the negative peak force.

6.2.6. Statistical analysis

The results obtained were tested at a 0.05 probability level and the combined effect of pressure and temperature was tested with a one-way analysis of variance (ANOVA), followed by a multiple comparisons test (Tukey HSD) to identify statistical significant differences between samples.

6.3. Results and discussion

In this study, lactic acid fermentation was performed under different conditions of pressure and temperature and the physicochemical and microbiological characteristics of each final yogurt were analyzed. Pressures of 10 and 30 MPa and temperatures of 35, 43 and 50 ºC were chosen since lactic acid fermentation occurred within a reasonable time to allow experiment execution and data generation, within not too long experimental times as observed in Chapter V. Fermentation times

were adapted according to the temperature used, i.e. fermentations at 35 and 50 ºC were slower and thus 24 hours were necessary to obtain yogurt, while fermentation was faster at 43 ºC and 10 hours were enough to obtain yogurt as the final product. Fermentation at atmospheric pressure (0.1 MPa) at the three studied temperatures was used as control.

6.3.1. Influence of fermentation conditions on the final pH, substrate consumption and product formation

In the beginning of this work, the obtained yogurts were characterized regarding the product formation and substrate consumption. For that, the final pH and respective sugars and lactate contents were measured, being the obtained results presented in Table 6.1.

Table 6.1. Final pH, lactose consumption and lactate productivity (Q_P) of the yogurts produced under different pressure (0.1, 10 and 30 MPa) and temperature (35, 43 and 50 ºC) conditions.

Fermentation conditions		Final pH	Lactose consumption (%)	Q_P (mg g^{-1} h^{-1})
Temperature	Pressure			
35 ºC	0.1 MPa	4.07 ± 0.06	45.8 ± 0.9	0.389 ± 0.017
	10 MPa	4.12 ± 0.01	50.4 ± 0.4	0.410 ± 0.032
	30 MPa	4.65 ± 0.02	52.6 ± 3.7	0.362 ± 0.032
43 ºC	0.1 MPa	4.12 ± 0.03	58.2 ± 0.0	1.074 ± 0.000
	10 MPa	4.17 ± 0.01	58.4 ± 1.2	1.087 ± 0.008
	30 MPa	4.58 ± 0.13	59.4 ± 1.8	1.060 ± 0.138
50 ºC	0.1 MPa	4.06 ± 0.04	65.5 ± 0.9	0.497 ± 0.000
	10 MPa	4.00 ± 0.03	65.7 ± 0.9	0.502 ± 0.003
	30 MPa	4.90 ± 0.01	65.5 ± 0.1	0.475 ± 0.001

Note: Results presented are average ± standard deviation.

One of the most important physicochemical parameters in yogurt production is pH, since the yogurt production process is complete, by definition, when a pH of 4.7 is reached (corresponding to the average isoelectric point for caseins) (Hui et al., 2012). Taking this into consideration, yogurt was obtained at almost all conditions tested in this work, except for fermentation at 30 MPa/50 ºC, where a slightly higher final pH of 4.90 was obtained within the fermentation time. In addition, the final pH of the products also gives information about the fermentative rate, since higher pH values correspond to lower fermentative rates. Therefore, the similar pH values ($p > 0.05$) obtained after fermentations at 0.1 and 10 MPa for all the temperatures tested indicates that the process occurred at similar fermentative rates. In contrast, higher pH values were observed at 30 MPa ($p < 0.05$), which corresponded to lower fermentative rates than the other pressure conditions tested. These results are in accordance with Mota et al. (2015), since pressure increase led to a decrease of the lactic acid

fermentation rate. However, the results obtained for fermentation at 30 MPa were different from the ones obtained in Chapter V, which can be due to a different preservation state of the starter cultures. In fact, the starter cultures used in this work were close to the end of shelf-life (one month until the expiration date), while the starter cultures used in Chapter V had a longer shelf-life (more than 6 months until the expiration date). This difference may influence the bacteria characteristics and, subsequently, influence their fermentative potential, decreasing the pressure resistance.

Lactose consumption and lactate productivity (Q_P) were determined taking into account the sugar and lactate concentrations of the final yogurts, respectively. Different effects on both parameters were observed when changing the fermentative conditions, i.e. pressure and temperature. At the same pressure, lactose consumption increased with temperature increase, while at the same temperature no significant differences were observed within the pressure range analyzed. An exception was observed at 35 ºC where the lactose consumed increased as the pressure increased, which may be explained by the higher energy requirements of microorganisms to trigger adaptation mechanisms in order to be able to withstand the higher pressure levels. In fact, this increase was only observed in lactose consumption and not in the lactate production, supporting the hypothesis outlined above.

As expected, higher Q_P values were observed for fermentations at 43 ºC, mainly due to the lower fermentation time needed to obtain yogurt as a final product. Analyzing the pressure effect on Q_P, higher values were obtained at 10 MPa for all incubation temperatures tested, which can be explained by metabolic changes occurring in the starter cultures, increasing the efficiency of the conversion of lactose into lactate under pressure, when compared to the respective fermentative process at 0.1 MPa. In contrast, fermentations at 30 MPa presented lower Q_P values, as expected by the higher pH values obtained in these cases.

In summary, the different fermentation conditions used in this work led to yogurt production in the end of fermentation time. Thus, in order to understand the effect of pressure and temperature variation on the yogurt characteristics, each final product of fermentation was analyzed regarding its microbiological and physical properties, which will be discussed in the next sections.

6.3.2. Influence of fermentation conditions on the starter cultures present in the final yogurt

Regarding the microbiological properties, the inoculum used in this work is composed by *S. thermophilus* and *L. bulgaricus*, corresponding to the starter cultures commonly used for yogurt production. Additionally, the finished product of lactic acid fermentation must contain live lactic acid bacteria (LAB) in amounts $\geq 7 \log_{10}$ (CFU g^{-1}) at the time of manufacture that should remain active at the end of the stated shelf-life, in order to be considered as a “live and active culture yogurt” (WHO/FAO, 2003). Thus, analysis of the starter cultures viability at the end of the fermentative process is preponderant to classify the final product obtained as yogurt. In addition, the

microbiological analysis also provides information about the starter's growth during fermentation and their resistance to the different conditions of pressure and temperature used during this work. Therefore, microbial counts of the starter cultures were performed at the beginning and end of fermentation, with the results obtained presented in Figure 6.1.

In general, both initial and final samples presented higher counts of *S. thermophilus* than *L. bulgaricus*. Additionally, almost all final samples contained the minimum amount of live LAB stablished for the yogurt samples, with the exception of fermentation at 50 ºC where microbial counts of 3.03, 6.55 and 6.51 $\log_{10}$ (CFU g^{-1}) were obtained after fermentations at 0.1, 10 and 30 MPa, respectively. Higher microbial counts were achieved after fermentations at 35 and 43 ºC for all pressures tested, with values higher than 8.00 $\log_{10}$ (CFU g^{-1}).

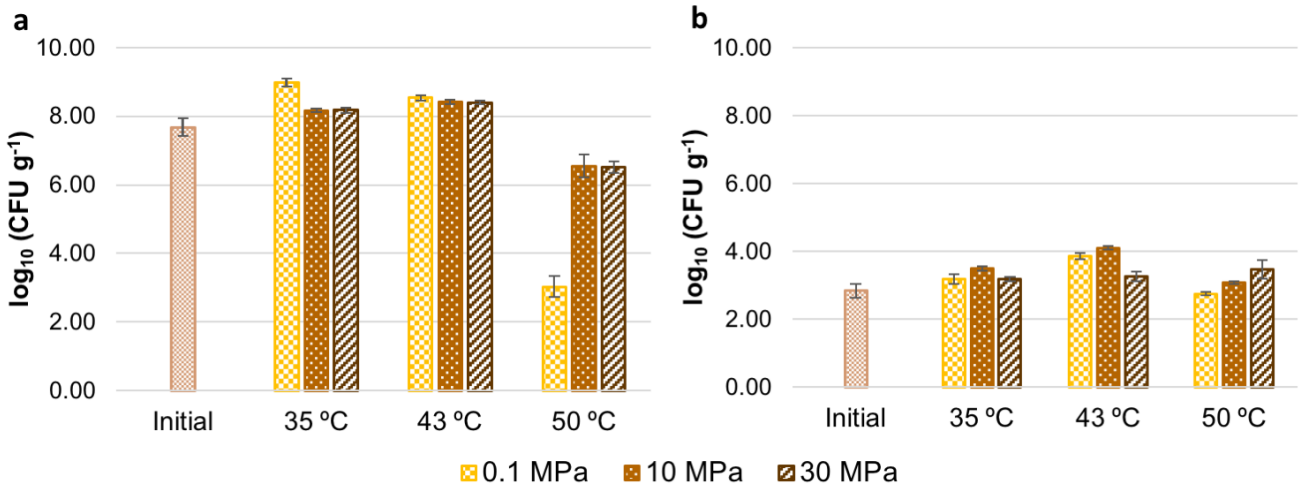

Figure 6.1. Microbial counts of *S. thermophilus* (a) and *L. bulgaricus* (b) of the yogurts produced under different pressure (0.1, 10 and 30 MPa) and temperature (35, 43 and 50 ºC) conditions.

Comparing the initial samples with the final ones, both bacteria counts increased during fermentation at 35 and 43 ºC, but different profiles were observed for each starter culture. While *S. thermophilus* presented significantly higher microbial counts at 0.1 MPa ($p < 0.05$), with similar values observed after fermentation under pressure (10 and 30 MPa, $p > 0.05$), significantly higher *L. bulgaricus* counts were obtained at 10 MPa ($p < 0.05$), with fermentation at 30 MPa presenting the lowest values. On the other hand, fermentations at 50 ºC presented some differences since the values remained in the same order of the initial sample, or even decreased, during the fermentation. This effect was more pronounced for *S. thermophilus* with microbial counts decreasing up to 4.65 $\log_{10}$ (CFU g^{-1}) in the worst case (fermentation at 50 ºC/0.1 MPa). Since fermentation occurred in these cases, the variations of the microbial counts can only be explained by the low cell resistance to high temperatures during the stationary phase. In fact, when cells are subjected to high temperatures for a relative long time, there is an increment of membrane permeability, leading to its disruption and cell

death (Chandler, 2017; Winter and Jeworrek, 2009). On the other hand, pressure decreases the membrane permeability due to the reduction of its fluidity (Winter and Jeworrek, 2009) and may also trigger the production of heat-shock proteins that protect the cells against heat and pressure (Abee and Wouters, 1999; Welch et al., 1993), which may support the fact that significantly higher counts were obtained after fermentations under pressure ($p < 0.05$), when compared to the fermentation at 0.1 MPa. Therefore, high pressures (10 and 30 MPa) may promote an increase of the cell resistance towards fermentation at higher temperatures, i.e. 50 ºC, relatively to fermentation at 50 ºC/0.1 MPa.

Comparing with Mota et al. (2015), similar results were obtained for both LAB after fermentations at 43 ºC. Therefore, despite the different inoculum used in this work (commercial lyophilized cultures were used in this work while a commercial yogurt was used by Mota et al. (2015)), the yogurts produced contained similar microbial counts at the end of fermentation, with higher *S. thermophilus* counts after fermentation at atmospheric pressure, contrasting to *L. bulgaricus* counts that presented higher values after fermentation under pressure. These results suggest that pressure influence on microbial growth seems to be independent of the inoculum type, as long as the initial sample contains a higher amount of *S. thermophilus* than *L. bulgaricus*.

Overall, the combination of different temperatures and pressures influenced the microbial growth of both starter cultures, with *S. thermophilus* being more sensitive to high temperatures and pressures than *L. bulgaricus*. However, with the exception of fermentation at 50 ºC/0.1 MPa, all conditions tested can be used to produce yogurts, according to the parameters indicated by WHO/FAO (2003), since lactic acid bacteria were present at the required amounts.

6.3.3. Influence of fermentation conditions on the yogurt syneresis

Syneresis is one of the most important structural feature of set type yogurt, corresponding to the accumulation of whey on the yogurt surface (Akalın et al., 2012; Bahrami et al., 2013). In the consumers point of view, acceptance is usually affected by syneresis, representing one of the major visible defects on the yogurt quality (Gündoğdu et al., 2009; Purwandari et al., 2007; Salvador and Fiszman, 2004). This physical phenomenon is related to the structural interactions present in the yogurt gel and its ability to retain whey, since the rearrangement of the three-dimensional protein network during yogurt formation leads to gel shrinkage and may result in the whey expulsion from the gel matrix (Bahrami et al., 2013; Lee and Lucey, 2003). Therefore, the quantitative evaluation of the whey separation provides information about not only the yogurt aspect, but also about the physical stability of the casein network (with higher syneresis values corresponding to a lower gel stability) (Lucey, 2002)

Figure 6.2 shows the results obtained for the amount of syneresis measured for the different yogurt samples. In general, similar syneresis values were observed for the yogurts produced at 0.1 and 10 MPa ($p > 0.05$), while an increase ($p < 0.05$) was verified when the pressure increased to 30 MPa, for all the temperatures tested (increased 7.20, 9.33 and 6.72 % at 35, 43 and 50 ºC, respectively,

relatively to the correspondent fermentations at 0.1 MPa). Thus, low pressures appear not to influence the amount of syneresis, although, by increasing the fermentation pressure, the yogurt gel became more unstable, and slightly higher amounts of whey were expelled after centrifugation, showing that these pressure ranges have a different effect on the physical structure of yogurt. In fact, Lee and Lucey (2004) demonstrated that the fermentative rate is one of the driving forces for whey separation, with slower fermentations leading to the formation of weaker gels, with a less developed protein network and fewer protein cross-links. These gels are more susceptible to the occurrence of rearrangements in the protein network, which are associated with an enhanced whey separation. Therefore, the slightly lower fermentative rates observed at 30 MPa can be behind this increase on the syneresis values.

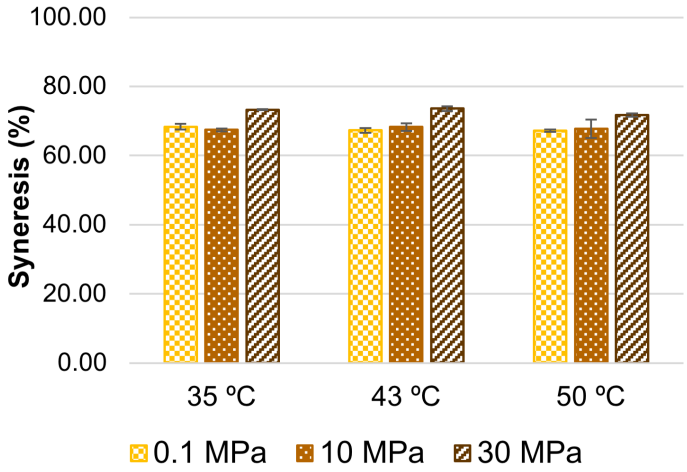

Figure 6.2. Syneresis of the yogurts produced under different pressure (0.1, 10 and 30 MPa) and temperature (35, 43 and 50 ºC) conditions.

Interestingly, several studies reported the influence of temperature on yogurt syneresis and lower syneresis values were achieved when lower fermentation temperatures were used (Abbasi et al., 2009; Lee and Lucey, 2004, 2003; Lucey, 2002; Lucey et al., 1998a; Nguyen et al., 2014), what was explained by the limited rearrangements in protein networks at lower temperatures, that probably led to a lower whey separation. However, in the present work, this effect of fermentation temperature was not detected, with the temperatures tested producing a yogurt with similar whey separation. As observed by Nguyen et al. (2014), parameters such as the milk type, the acidification rate and the protein content may influence the physical properties of yogurt gels, in addition to the fermentation temperature, thus, as a consequence, different results may be obtained for different yogurt preparations.

Therefore, in this case, pressure seems to have a higher influence on yogurt syneresis than fermentation temperature. Comparing the yogurts fermented under pressure, yogurts produced at 10 MPa are, potentially, more likely to have a higher consumer acceptance (at the same level as the yogurts produced at control conditions) than yogurts fermented at 30 MPa.

6.3.4. Influence of fermentation conditions on the yogurt texture

Texture was another parameter analyzed in this work, since this is one of the main characteristics that define yogurt quality, affecting its appearance, mouthfeel and the overall acceptability of the final product (Kroger, 1976). Since yogurt is a non-Newtonian pseudoplastic material with a highly time dependent behavior (Basak and Ramaswamy, 1994; Benezech and Maingonnat, 1994), the measurement of its rheological properties have poor reproducibility and high sensitivity to the production process (Yoon and McCarthy, 2002), making it difficult to use in routine quality control (Ares et al., 2006). Thus, the use of empirical or imitative methods (such as, penetration tests and texture profile analysis) have been preferred to measure the textural properties of yogurt, due to its simplicity, affordability and the good correlation with sensorial properties of the final product (Benezech and Maingonnat, 1994; Hellinga et al., 1986). In fact, this texture analysis can be easily used by manufacturers to adjust the formulation and control the production process to achieve a final product with a higher acceptability by the consumers (de Abreu et al., 2016). However, these empirical methods have some drawbacks, since relative scales are used, with the results obtained being characteristic for a given set of experiments, making it barely impossible to compare results between different experimental conditions (Benezech and Maingonnat, 1994).

Here, a penetration test was performed to each yogurt sample, with the force-response being monitored as a function of penetration depth and experimental time. Figure B.1 in Appendix B shows typical penetration curves acquired for each yogurt produced. Differences between the force *vs.* distance/time profiles can be observed, which are related to the expected effects of pressure and temperature upon the yogurt's structure and physical properties.

In general, higher force peak values were observed in the yogurts fermented at 0.1 MPa, when compared to the ones fermented under pressure, which were converted to significant higher ($p < 0.05$) values of firmness, as showed in Figure 6.3a. Despite several factors can affect the physical properties of yogurt, proteins appear to play a major role in yogurt texture, with higher protein content leading to higher firmness values (Domagała, 2009; Salvador and Fiszman, 2004; Yu et al., 2016). In addition, the interactions between whey proteins and caseins by hydrophobic and electrostatic interactions, and covalent thiol/disulphide bonds present in the gel network of yogurt, can be also responsible for the increase of gel firmness (Steventon et al., 1990; Vasbinder et al., 2003). Since the yogurts produced in this work only differ on the fermentation conditions, the differences in texture can only be due to the differences in the protein interactions, promoted by the variation of pressure and temperature, directly and/or indirectly, through the effect on the bacteria.

In fact, one of the main effects of pressure on cell components is the modification of the protein structure and functionality, due to weakening of electrostatic and hydrophobic interactions, and the thiol/disulphide exchange reactions (Funtenberger et al., 1997; Heremans and Smeller, 1998). Therefore, the lower firmness of yogurts produced under pressure may be explained by the lower protein interactions in the gel network produced under pressure, when compared to yogurts fermented at 0.1 MPa.

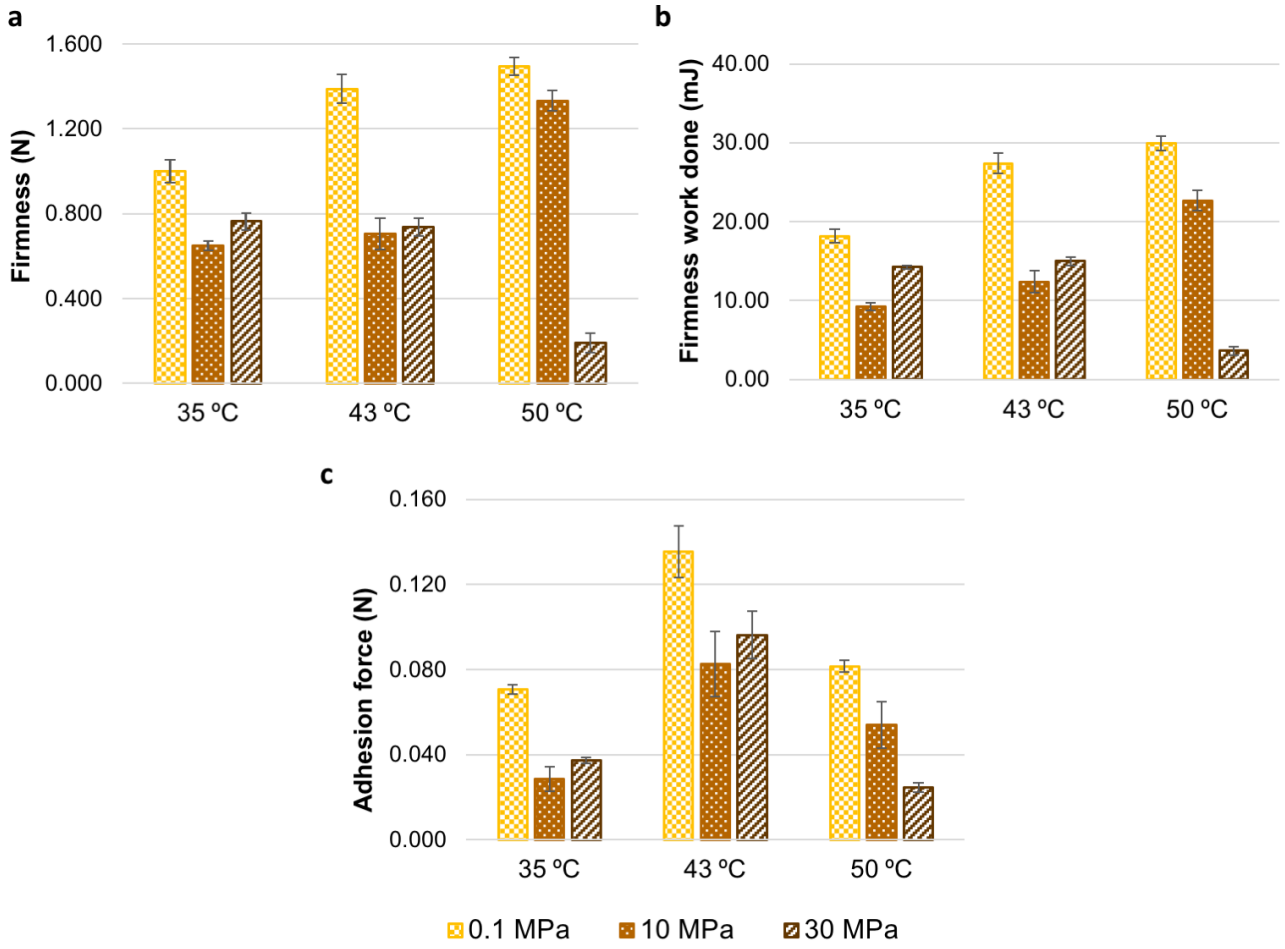

Figure 6.3. Textural parameters (firmness (a), firmness work done (b) and adhesion force (c)) of the yogurts produced under different pressure (0.1, 10 and 30 MPa) and temperature (35, 43 and 50 ºC) conditions.

On the other hand, the increase of pressure between 10 and 30 MPa did not show a clear tendency in its effect on yogurt texture: while at 35 ºC, the firmness increased with pressure, increasing from 10 to 30 MPa (i.e., 0.666 ± 0.023 to 0.750 ± 0.040 N, $p < 0.05$), similar values of firmness were obtained under pressure at 43 ºC (0.654 ± 0.074 and 0.728 ± 0.041 N for 10 and 30 MPa, respectively, $p > 0.05$), and the firmness decreased with the pressure increasing at 50 ºC (1.058 ± 0.049 and 0.230 ± 0.044 N for 10 and 30 MPa, respectively, $p < 0.05$). The combined effect of pressure and

temperature can explain these differences, since different combinations can result in different levels of protein denaturation, which is strongly related to the gel firmness of yogurt (Dannenberg and Kessler, 1988). Another parameter described in literature that may influence the gel firmness of yogurt is the final pH (Harwalkar and Kalab, 1986), with lower pH values presenting higher firmness values (increase of 20 % when the final pH decreased from 4.50 to 3.85). The authors assumed that the increase of the positive charge of casein due to lower pH (below the isoelectric point of caseins) led to a higher intramicellar repulsion, resulting in the casein swelling that increased the rigidity of the yogurt gel. Therefore, both fermentation conditions may possibly influence the protein interactions within the gel network, resulting in different firmness levels for each combined condition tested.

Regarding the temperature effect, fermentation at 50 ºC presented the highest firmness values at 0.1 and 10 MPa (1.453 ± 0.042 and 1.058 ± 0.049 N, respectively) and the lowest value at 30 MPa (0.230 ± 0.044 N). In fact, firmness showed a tendency to increase with temperature increase when low pressures were used (i.e., 0.1 and 10 MPa), but when pressure increased to 30 MPa, a tendency to decrease with the temperature increase was observed. Interestingly, except at 30 MPa, these results are the opposite of the expected, since temperature increase may lead to a decrease of firmness due to the higher acidification rate that results in lower number of protein interactions (Sodini et al., 2004). On the other hand, the final product with a weak and thin body obtained after fermentation at 30 MPa/50 ºC can be explained by the low acidity and high incubation temperature used (Bodyfelt et al., 1988).

In addition, the firmness work done was also estimated for these final products (Figure 6.3b), which corresponds to the energy required to drive the probe during the downward penetration step. As expected, the values obtained followed the same profile of the firmness values: higher values for fermentations at 0.1 MPa when compared to fermentations performed under pressure, and increasing with temperature increase, with the exception of fermentation at 30 MPa/50 ºC.

Adhesion force, which corresponds to the force necessary to detach the probe from the sample, was also evaluated (Figure 6.3c). The obtained values were very low, indicating that the final yogurts poorly adhere to surfaces. Regarding the effect of pressure and temperature on this property, no conclusion was possible to be drawn since no clear tendency was observed in the results obtained for each yogurt produced. In fact, these results present a lower significance since the values obtained are lower than the load cell accuracy (0.250-0.500 N), which corresponds to 0.5-1.0 % of the load cell capacity (5 kg).

The penetration test used for the assessment of the texture properties of the yogurts produced was able to show some tendencies about the combined effect of pressure and temperature on the yogurt gel firmness. For instance, lower firmness values were achieved for yogurts produced under pressure for all temperatures tested, which can represent an advantage to the production process since an

excessive firm texture can be considered as a defect by consumers, which expect a smooth, fine-bodied product (Bodyfelt et al., 1988).

6.4. Conclusions

Overall, pressure and temperature when applied to lactic acid fermentation, influence the final yogurt characteristics and, possibly, the consumer perception. In addition to the fermentative kinetics, fermentation conditions also affect the microbial growth of the starter cultures and the physical characteristics of the final product. For instance, both *S. thermophilus* and *L. bulgaricus* showed different sensitivities to the different conditions used in this work, with *S. thermophilus* being more sensitive to the combination of high temperatures and high pressures than *L. bulgaricus*. On the other hand, physical characteristics of yogurts were assessed by the measurement of syneresis and texture, with different conditions of fermentation resulting in yogurts with different physical properties, in what regards to whey separation and firmness after fermentation under pressure. Between the yogurts produced under pressure, the ones produced at 10 MPa are more likely to have a higher consumer acceptance since they presented a lower whey separation and a firm texture, without being excessive. Therefore, the variation of pressure and temperature during lactic acid fermentation may be used to modulate both the process and the yogurt characteristics, in order to improve the yogurt quality and the consumers' acceptance. However, this study was only a preliminary study, thus a further study about the rheological and sensorial properties should be performed to fully characterize the pressure and temperature influence on the final yogurts.

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CHAPTER VII

Comparative metabolomic profiling of yogurts produced under different conditions of pressure and temperature

7.1. Introduction

Fermentation under non-conventional conditions has gained prominence in the last years, since the variation of the fermentation conditions can bring novel characteristics and features not only to the fermentative process, but also to the final products of fermentation (Mota et al., 2018). When microbial cells are exposed to mild stress conditions during growth and fermentation, the activation of general and specific stress response mechanisms occurs, resulting in the coordinated expression of genes responsible for the cellular processes regulation, in order to improve the stress tolerance (Huang et al., 2014; Storz and Hengge, 2000; van de Guchte et al., 2002). As a consequence, several metabolic activities are affected, which will be reflected in the metabolome of the fermentative microorganisms, along with the bioproducts and on the bioprocess itself (Serrazanetti et al., 2009).

The study of metabolome is only possible nowadays due to the great technological progress in instrumental analysis, which enable the development of metabolomic approaches (Tomita et al., 2018). By definition, metabolomics is a research field that involves the characterization, including identification and quantification, of the complete collection of small molecule metabolites in a determined biological system (Johanningsmeier et al., 2016). The combination of comprehensive compositional analysis by high-throughput analytical instruments with multivariate statistical analysis allows the generation of new knowledge about the compositional similarities and differences between defined groups of samples (Johanningsmeier et al., 2016; Tomita et al., 2018) As a consequence, metabolomics is playing an increasingly important role in systems biology, being now used for many applications, such as microbiology, diagnostic biomarker discovery, toxicological testing, food and beverage analysis, plant and animal phenotyping, and drug discovery and development (Mozzi et al., 2013; Sugimoto et al., 2012). Regarding food analysis, the complete collection of small compounds present in foods is commonly called food metabolome, and a large number of these compounds can be simultaneously identified and quantified in a single analysis using the metabolomic approach (Fiehn et al., 2000). Therefore, any change in the food metabolome caused by microorganisms, processing, storage and chemical contamination can be rapidly detected, which constitute a significant improvement for the assessment of both food adulteration and food quality (Johanningsmeier et al., 2016; Mozzi et al., 2013; Wishart, 2008).

However, a single analytical technique to comprehensively study the entire food metabolome is not yet readily available, given the great chemical diversity of the compounds present in food matrix (including sugars, fatty acids, peptides, amino acids, organic acids, vitamins, polyphenols, minerals, among others) (Wishart, 2008). The technologies primarily used for metabolomic studies include nuclear magnetic resonance (NMR), liquid chromatography–mass spectrometry (LC-MS), and gas chromatography–mass spectrometry (GC-MS) (Johanningsmeier et al., 2016). But, each of these techniques has advantages and limitations, as represented in Figure 7.1. In the case of LC-MS and GC-MS, the chemicals are separated by chromatography and the high-

resolution MS accurately measure their mass-to-charge ratios and abundance (Jones et al., 2012; Patti et al., 2012). On the other hand, NMR is one of the most commonly used analytical techniques in metabolomic studies, measuring the molecules response to radiofrequency *stimuli* by chemically distinct atomic nuclei in a magnetic field to provide information about the structure and dynamics of molecules (Patti et al., 2012; Reo, 2002). Despite the lack of sensitivity of NMR-based techniques when compared to MS-based strategies, they present the most uniform detection between samples (De Vos et al., 2007).

		Advantages	Disadvantages
NMR		• Rapid and robust analysis; • No separation or derivatization; • Detects all organic classes; • Large pool of software and databases for metabolite identification.	• Limited sensitivity; • Large instrument footprint.
LC-MS		• Excellent sensitivity; • Flexible technology; • Potential for detecting largest portion of metabolome.	• Less robust than GC or NMR; • Poorer chromatographic resolution than GC.
GC-MS		• Sensitive; • Excellent separation capability; • Large pool of software and databases for metabolite identification.	• Requires compounds that are volatile or made volatile via chemical derivatization.

Figure 7.1. Advantages and limitations of the most widely used metabolomic technologies: NMR, nuclear magnetic resonance; LC-MS, liquid chromatography-mass spectrometry; and GC-MS, gas chromatography-mass spectrometry. Adapted from Johanningsmeier et al. (2016).

The metabolomics study can use two different approaches depending on the scope of the metabolic profiling: an untargeted or a targeted approach. The untargeted approach (also known as chemometric approach) focuses on the detection of metabolite groups without identifying or quantifying a specific compound, only with the purpose of the identification of their spectral patterns or fingerprints (Cevallos-Cevallos et al., 2009; Sun and Hu, 2016). In order to identify the relevant spectral features that distinguish samples classes, statistical comparisons are performed usually using an unsupervised clustering (Principal Component Analysis or PCA) or a supervised classification (Partial Least Squares Discriminant Analysis or PLS-DA) (Wishart, 2008). On the other hand, the targeted approach (also known quantitative metabolomics) focuses on the identification and quantification of as many metabolites in the sample as possible (Cevallos-Cevallos et al., 2009; Wishart, 2008). Then, the obtained data is also statically

processed (with PCA or PLS-DA) in order to identify the most important biomarkers or informative metabolic pathways (Weljie et al., 2006).

Overall, the study of metabolite profiling in fermented foods can be used to observe the metabolite changes during fermentation and to possibly predict some of the properties of the fermented product, including sensorial and nutritional characteristics, among others (Mozzi et al., 2013). Therefore, this work intends to study the influence of the fermentation conditions on the metabolome of the resultant yogurts, and possibly disclose some adaptation mechanisms to the stressful conditions. For that, yogurt was produced under different conditions of pressure (10 MPa and 0.1 MPa) and temperature (35, 43 and 50 ºC) and the metabolite abundance in each yogurt sample was profiled by ^{1}H NMR spectroscopy, using a non-targeted strategy. This analytical technique was selected for this study since it is simple and fast, requiring minimal sample preparation. In addition, it allows the simultaneous detection of primary and secondary metabolites and the identification of a high number of metabolites (Kim et al., 2011), which could be an advantage for this preliminary metabolomics study.

7.2. Material and methods

7.2.1. Yogurt production

Milk preparation was performed based on Settachaimongkon et al. (2014) and Haque et al. (2001), with reconstitution of 10 % (w v^{-1}) Nido whole milk powder (Nestlé, Portugal) in distilled water to obtain a final liquid milk with approximately 9.7 % dry matter content. The prepared milk was pasteurized at 90 ºC for 20 minutes in a circulating water bath and it was then cooled rapidly to ambient temperature by immersion in running tap water. Thereafter, milk was stored overnight at 5 ºC.

Sample preparation consisted in the combination of the pasteurized milk with a lactic acid culture (Yo-Aktiv of ADMIX Ltd., Bulgaria, composed by *Lactobacillus bulgaricus* and *Streptococcus thermophilus*) at a concentration of 2 g L^{-1}, accordingly to the manufacturer's instructions. After homogenization, the mixture was transferred to heat sealed polyamide–polyethylene bags (PA/PE-90, Plásticos Macar – Indústria de plásticos Lda., Portugal). All the bags were sterilized by UV-light (for 15 min) in a laminar flow cabinet (BioSafety Cabinet Telstar Bio II Advance, Spain) before being filled with the mixture.

The mixture was then incubated at different pressure and temperature conditions. The experiments were carried out using high pressure equipment (FPG7100, Stanstead Fluid Power, United Kingdom). The equipment has a pressure vessel of 100 mm inner diameter and 250 mm height surrounded by an external jacket to control the temperature. A mixture of propylene glycol and water (40:60 v/v) was used as pressurization fluid.

Fermentations were performed at 10 MPa and three different temperatures (35, 43 and 50 ºC), using fermentation under atmospheric pressure (0.1 MPa) at each temperature as a control.

According to the fermentative rates for each temperature used, fermentation times were adapted: 24 hour fermentation was used at 35 and 50 ºC, while 10 hours were used at 43 ºC. The final product of each fermentation was collected and stored at -20 ºC. Each experiment was performed in duplicate.

7.2.2. Nuclear Magnetic Resonance experiments

Extracts were prepared by centrifugation (at 10,000 *g* for 15 minutes) followed by filtration (0.45 μm pore diameter) of the supernatants. Extracts were then dried in a vacuum centrifuge, followed by storage in a desiccator until NMR analysis. Before NMR spectral acquisition, samples were reconstituted using 600 μL of phosphate buffer (100 mM, pH 3.0) containing 0.01 % of 3-(trimethylsilyl)propionic-2,2,3,3-*d4* acid, sodium salt (TSP-*d4*) as a chemical shift and intensity reference. The mixture was then centrifuged (4500 *g*, 25 °C, 5 min) and transferred into 5 mm NMR tubes to be analyzed.

^{1}H NMR spectra were recorded at 300 K on a Bruker Avance DRX 500 spectrometer (Bruker BioSpin, Germany), operating at a proton frequency of 500.13 MHz, equipped with an actively shielded gradient unit with a maximum gradient strength output of 53.5 G cm^{-1} and a 5 mm inverse probe. For each sample, a 1D ^{1}H NMR spectrum was acquired using the *noesypr1d* pulse sequence (Bruker pulse program library) with water presaturation. For all spectra, 128 transients were collected into 32,768 (32 K) data points with a spectral width of 10000 Hz, an acquisition time of 3.3 s and relaxation delay of 5 s. Each free induction decay (FID) was zero-filled to 64 k points and multiplied by a 0.3 Hz exponential line-broadening function prior to Fourier transformation. iNMR software was used to manually phase and baseline correct the spectra. The spectra were exported as a matrix using R-Studio in-house scripts and subsequently normalised to TSP. The spectra were overlaid and checked in iNMR to see whether alignment was required. If required, the speaq package was used in R software.

7.2.3. Multivariate data analysis

The multivariate analysis were applied to the aligned spectra, using the ropls package (Thévenot et al., 2015) in R software. Differences among samples were visualized by Pareto-scaled for principal component analysis (PCA). The identification of relevant metabolites was carried out by comparing the spectra with those of standard compounds from the Biological Magnetic Resonance Data Bank, the Human Metabolome Database and the Chenomx NMR Suite software. The relative amounts of the NMR metabolites and the effect size were determined by integrating the area under the most well-separated metabolite peak using in-house R scripts. Pairwise t-tests were carried out using the False Discovery Rate (FDR) to adjust for multiple testing. Effect sizes were calculated and corrected for small sample sizes using the formula:

$$Effect\ size = \left(1 - \left(\frac{3}{(4n1 + n2 - 2) - 1}\right)\right)\left(\frac{x1 - x2}{pooled\ SD}\right)$$

where pooled SD is the pooled standard deviation, x1 and x2 are the mean levels of metabolite *x* and *n1* and *n2* are the number of replicates.

7.3. Results and discussion

Fermentation at 10 MPa resulted in higher fermentative rates, higher lactate productivities, and higher yields and efficiencies when compared to the fermentation at 30 MPa (as described in Chapter V). On the other hand, the physical characteristics of the yogurts produced at 10 MPa are more similar to the yogurts produced at 0.1 MPa, as well as the amount of starter cultures present in the final yogurt (as described in Chapter VI). Therefore, the yogurts produced at 10 MPa were selected to analyze their general biochemical composition by ^{1}H NMR and to study the differences between samples caused by the variation of both pressure and temperature during fermentation. The temperatures used (35, 43 and 50 ºC) were selected due to the reasonable time needed to allow the experiment execution and data generation, as explained in Chapter VI. In addition, fermentation at atmospheric pressure (0.1 MPa) and at the respective temperature was used as control.

The characteristic 1D ^{1}H NMR spectra of the 6 yogurt samples are shown in Figure 7.2, with each fermentation condition identified. Before peak identification, a code name was assigned to each peak (M1 and M66) ranging from high to low ppm, as presented in Table C.1 in Appendix C. In order to try to identify some of the metabolites present in the yogurt samples, spectral comparisons with databases was performed and the results obtained are summarized in Table 7.1. We attempted to identify the metabolites characteristic of lactic acid fermentation and also those that present changes between samples during the data processing.

Table 7.1. List of relevant metabolites identified in the samples by comparison with databases and an appropriate software, with the respective chemical shifts and code names (attributed between M1 and M66, from higher to lower ppm values).

Compounds	Chemical shift (ppm)	Code name
2,3-butanediol	1.12 – 1.16	M66
Acetate	1.92 – 1.95	M60
Acetaldehyde	2.03 – 2.08	M58
Acetoin	2.22 – 2.24	M55
Citrate	2.50 – 2.58; 2.67 – 2.75	M50; M49
Diacetyl	2.37 – 2.38	M53
Formate	8.44 – 8.46	M5
Lactate	4.11 – 4.17; 1.28 – 1.40	M36; M63
Pyruvate	2.42 – 2.45	M52

Regarding the full spectra of the different yogurts (Figure 7.2a), no obvious differences could be seen, with the peaks presenting higher intensity corresponding to lactate (mainly M63) and sugars (M41-M22; 3.0-5.5 ppm). These results corroborate those obtained in Chapter V, where the compounds present in higher amounts were the sugars present in milk (lactose, galactose and glucose) and lactate, the main product of lactic acid fermentation.

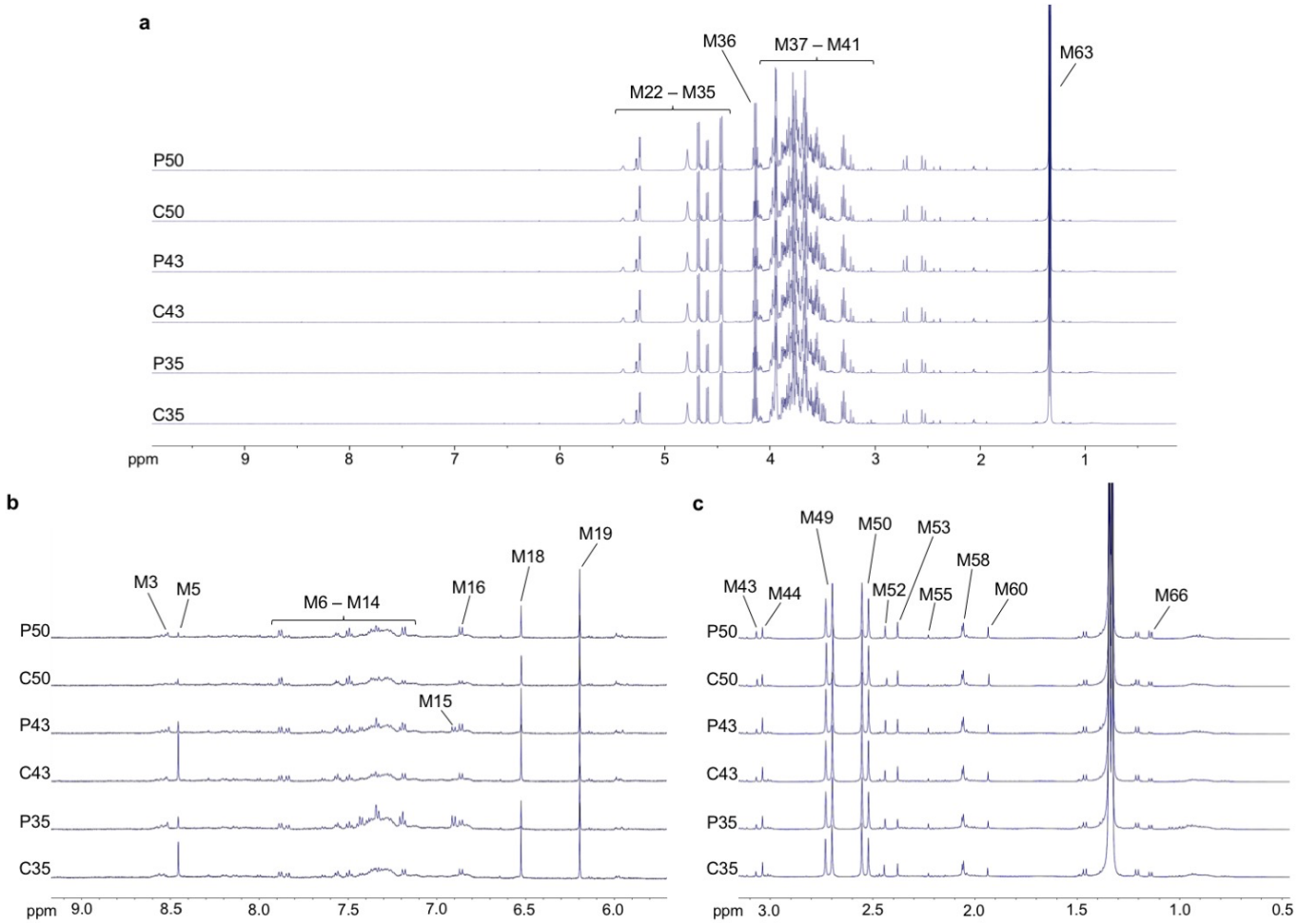

Figure 7.2. 500 MHz ^{1}H NMR spectra of yogurt produced under different conditions of pressure and temperature: (a) full spectra; and expansions for (b) aromatic region (5.8-9.0 ppm), and (c) aliphatic region (0.5-3.1 ppm). C35, C43 and C50 samples correspond to yogurts fermented at 0.1 MPa and 35, 43 and 50 ºC, respectively; P35, P43 and P50 samples correspond to yogurts fermented at 10 MPa and 35, 43 and 50 ºC, respectively. Signals were identified with a code name from M1 to M66, ranging from the higher to lower ppm.

In order to identify minor intensity peaks and to observe if they differ between the different yogurts, expansions in two different regions of the ^{1}H NMR spectra were performed. The obtained insets for the aromatic (5.8-9.0 ppm) and aliphatic (0.5-3.1 ppm) regions are also shown in Figure 7.2b and 7.2c, respectively, where the most differences were observed. For instance, the aromatic region (Figure 7.2b) showed the greatest number of differences between samples. This region for

the yogurt samples is characterized by the presence of peaks corresponding to formate, aromatic amino acids, such as phenylalanine, histidine, tryptophan and tyrosine (M16-M6; 6.8-8.0 ppm), and other peaks that could not be assigned (e.g, M19, M18 and M3; 6.2, 6.5 and 8.5 ppm). The aliphatic region (Figure 7.2c) is characterized by peaks corresponding to organic acids, alcohols and aliphatic amino acids, the main products of fermentation, including lactate, citrate, acetate, pyruvate, acetoin, 2,3-butanediol, diacetyl, among others. In this case, the differences observed between samples were not as pronounced as for the aromatic region, but different intensities were obtained for peaks identified as M66, M60, M55, M53, M44 and M43. Therefore, the fermentative conditions (namely, pressure and temperature) have an influence on the metabolism of starter cultures with the production of different proportions of metabolites characteristic of fermentation, resulting in different yogurts. These differences will be discussed in more detail below.

In order to identify the differences seen for samples fermented under different pressure and temperature conditions, a Principal Components Analysis (PCA) was carried out using a dataset generated from the full ^{1}H NMR spectra. PCA is an unsupervised statistical analysis, that is widely used as a first exploratory step in metabolomics studies. This statistical tool converts high dimensional data into fewer dimensions, maintaining as much variance from the original data as possible (Boccard et al., 2010; Nyamundanda et al., 2010). As a result, sample distribution in the principal component (PC) space is given by score plots, where the Euclidian distance between individual samples reflects the degree of the variation in metabolite profiles among samples and the loading plots describe the contribution of individual metabolites to each PC (Sugimoto et al., 2012). The scores plot obtained in this work is presented in Figure 7.3 and the respective loadings plots are shown in Supplementary Material (Figure S7.1). The PCA model showed a good fit ($R^2X = 0.564$), with the first and second principal components (PC1 and PC2) explaining 31 % and 25 % of the total variance, respectively.

The PCA scores plot revealed separation between control samples, while samples fermented under pressure were less separated. However, the reproductibility between replicas was in some cases of the order of the group separation. Therefore, differences in the metabolic profiles of control samples were greater than for samples fermented under pressure, possibly indicating that temperature had a higher influence on the yogurts produced at 0.1 MPa. In fact, variation of temperature does not have a major effect on yogurt when fermentation is performed under pressure, as explained in Chapter V.

Analyzing the loading plots, sugars are the main metabolites that positively contributed to PC1, while lactate (M63) is the main metabolite that negatively contributed (Figure C.1a in Appendix C). Taking this into consideration, it would be expected that samples fermented at 0.1 MPa/35 ºC (C35) and 10 MPa/35 ºC (P35) contained higher amounts of lactate, which it was not observed in the HPLC analysis performed in Chapter V. In contrast, the higher concentrations of lactate were obtained at 43 ºC for both 0.1 MPa and 10 MPa. On the other hand, both sugars and

lactate positively contributed to PC2 (Figure C.1b). In this case, the samples fermented at 0.1 MPa/43 ºC (C43) and 10 MPa/43 ºC (P43) showing higher PC2, indicating that these samples contained higher amounts of lactate and sugars, when compared to the other samples. These results are in accordance to the ones obtained in Chapter V, since a higher yield and fermentative efficiency was observed for these samples. However, both PC1 and PC2 presented lower contributions to sample discrimination, making difficult to disclose the differences between the metabolic profile of the different samples. Thus, this model had low significance, which may be due to high similarity between samples. For a model with higher significance, more replicas for each sample should have been used.

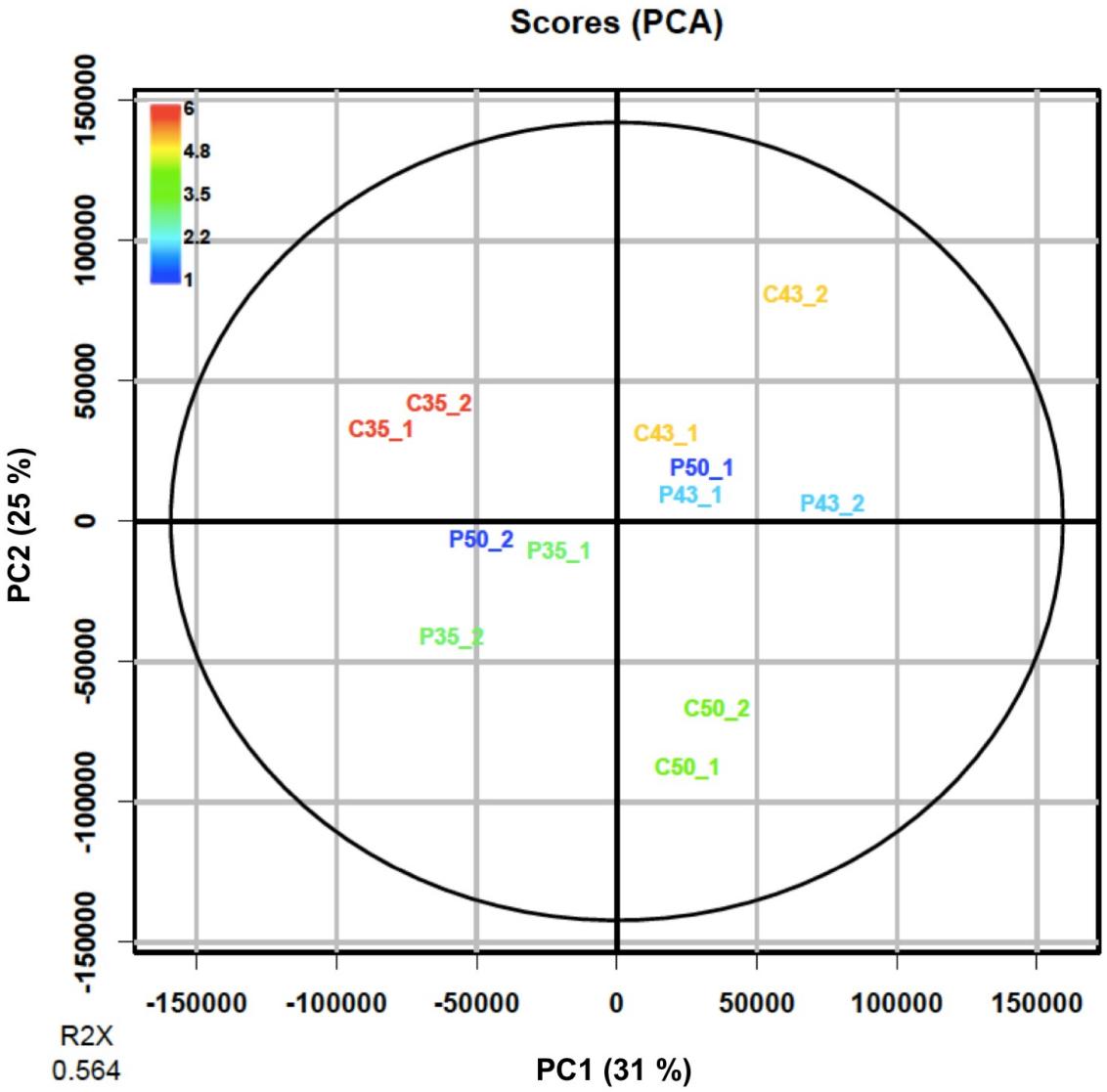

Figure 7.3. PCA scores plot of yogurt produced under different conditions of pressure and temperature, obtained by 1D ^{1}H NMR. C35, C43 and C50 samples correspond to yogurts fermented at 0.1 MPa and 35, 43 and 50 ºC, respectively; P35, P43 and P50 samples correspond to yogurts fermented at 10 MPa and 35, 43 and 50 ºC, respectively. In all cases, the results are presented in duplicated (indicated as 1 and 2).

In order to further study the differences in the metabolic profile of the samples fermented at 0.1 and 10 MPa, a PCA for each temperature was performed, being the obtained score plots presented in Appendix C (Figure C.2). Despite the good fits obtained (R^2X of 0.856, 0.901, 0.853 for 35, 43 and 50 ºC, respectively) and the higher contributions for PC1 and PC2 (PC1: 58 %, 60 %, 55 %; PC2: 28 %, 30 %, 30 %, for 35, 43 and 50 ºC, respectively), no significant differences between samples were observed, presenting no clear separation. Similarly, we carried out a partial least squares-discriminant analysis (PLS-DA) separating the samples by classes, i.e. C and P. PLS-DA is a regression-based method that uses additional information to adjust the model in order to capture the related variation into the original variables (Boccard et al., 2010). In metabolomics, this analysis is commonly used to sharpen the separation between groups or observations (Sugimoto et al., 2012). However, the results obtained were similar to those obtained with PCA, showing a no clear separation between samples. Therefore, more samples need to be prepared and analysed and/or more sensitive analytical technologies could be used to detect differences between these samples.

In a different approach, the differences between these samples were plotted in 3 different Volcano plots (Figure C.3 in Appendix C), one for each temperature. In these plots, the relationship between the effect size and p-values are showed, representing the differential abundances between the metabolites present in the samples fermented at 0.1 MPa and 10 MPa for each temperature tested, accordingly to their effect size. This representation shows the differences in the metabolite accumulation, indicating which metabolite was more accumulated for each sample. For instance, metabolites with signals present mostly in the aliphatic region were more accumulated in the samples fermented under pressure (M57 and M59 for 35 ºC; M55 for 43 ºC; M55 and M24 for 50 ºC), while the metabolites more accumulated in the control samples had signals at the sugar (M33 for 35 ºC) and aromatic (M4, M5 and M18 for 43 ºC, M4 for 50 ºC) regions. Therefore, these differences may be part of the stress response from cells to be able to overcome the higher pressure conditions that are subjected during these fermentation processes.

In addition, the volcano shape can give us information about the difference level between samples. For instance, the narrower the volcano in the Volcano plot, the more similar the samples are, and vice-versa. On the other hand, the points were also colored based on their fold change and statistical significance of each metabolite variance between samples. Black points indicates the metabolites without significant change, while the metabolites with increasing variation are colored from orange to green and red points, accordingly to their significance. Therefore, both yogurts fermented at 43 ºC presented a very similar metabolic profile due to the shape of the correspondent Volcano plot and the high amount of black points (Figure C.3b). In contrast, a different metabolite accumulation after fermentations at 35 and 50 ºC were observed between control and pressurized samples, with the presence of orange points (Figure C.3a and C.3c, respectively). These results are in accordance to the PCA score plot, where a better separation between control and pressurized samples was observed for samples fermented at both 35 and 50

ºC than fermentations at 43 ºC. Therefore, fermentation at 43 ºC results in identical yogurts both under pressure and at atmospheric pressure, which may be explained by a less complex adaptation process when only pressure is changed from the optimal growth conditions, since 43 ºC corresponds to the optimal temperature for yogurt production at 0.1 MPa. In contrast, it is possible that bacteria require a more complex adaptation process when both pressure and temperature are changed from the optimum, as occur in the other fermentation conditions tested in this work.

In order to semi-quantitatively compare the compositional changes between the yogurt samples analyzed, normalized areas of the compounds identified were calculated. Firstly, it was performed the identification of the signals correspondent to the metabolites present in the yogurt samples, which were already observed by HPLC analysis (Chapter V and VI). Since the sugars were present in high amounts, the identification of lactose, galactose and glucose was not possible due to the overlap of several signals in the sugar region (M22-M41 in Figure 7.2). However, the signals correspondent to lactate and citrate were identified, with both having two correspondent signals due to the protons in two different environments. For instance, M36 and M63 correspond to lactate, while M49 and M50 correspond to citrate. The respective normalized areas were calculated (Figure C.4 in Appendix C) and no significative variation between samples was observed. These results are in accordance to those obtained with HPLC in Chapter V and VI.

In addition to the lactate production, starter cultures are able to produce several other compounds in lower amounts that are responsible for yogurt flavor. In this case, pyruvate is used as a metabolic precursor of the mixed acid metabolism, whose metabolic pathways are represented and described in Chapter I (Chen et al., 2017; Walstra et al., 2005). By analysis of the spectra, signals correspondent to some of these compounds were identified, including pyruvate (M52), acetate (M60), formate (M5), acetaldehyde (M58), diacetyl (M53), acetoin (M55) and 2,3-butanediol (M66). The normalized areas of these compounds are presented in Figures 7.4 and 7.5.

Acetaldehyde corresponds to one of the most important flavor compound in yogurt, usually present in higher amounts than the other flavor compounds and gives the characteristic green apple or nutty flavor to the yogurt (Bodyfelt et al., 1988; Chaves et al., 2002). Comparing with the other flavor compounds identified, acetaldehyde presented the highest amounts, but no significant variation was observed between samples (Figure 7.4a). In lower amounts, acetate and pyruvate along with 2,3-butanediol were also identified (Figure 7.4b, 7.4c and 7.4d, respectively), corresponding to compounds that also influence the yogurt flavor and aroma (Cheng, 2010). In these cases, it was not possible to draw a conclusion about the influence of the fermentation conditions on their production, since both acids were apparently produced in higher amounts in samples fermented at 10 MPa/50 ºC (P50), and higher amounts of 2,3-butanediol were obtained after fermentation at 0.1 MPa/35 ºC (C35) and 10 MPa/50 ºC (P50). However, these effects may not be significative due to the higher standard deviation associated.

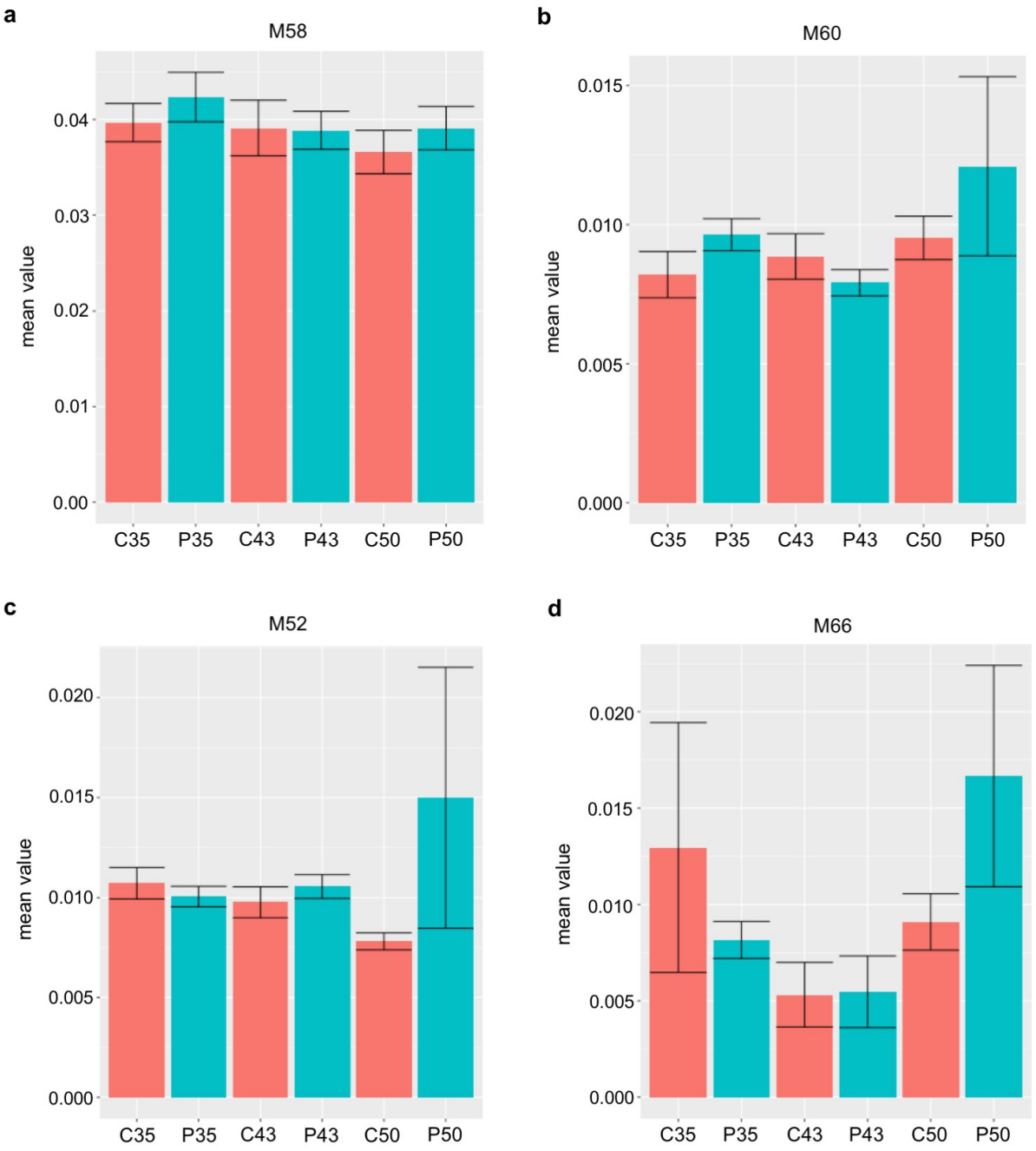

Figure 7.4. Metabolite plots showing the accumulation of acetaldehyde (M58, a), acetate (M60, b), pyruvate (M52, c) and 2,3-butanediol (M66, d) on yogurts produced under different conditions of pressure and temperature. C35, C43 and C50 samples correspond to yogurts fermented at 0.1 MPa and 35, 43 and 50 ºC, respectively; P35, P43 and P50 samples correspond to yogurts fermented at 10 MPa and 35, 43 and 50 ºC, respectively.

Interestingly, signals corresponding to diacetyl and acetoin showed different abundances between the analyzed yogurt samples, as can be observed in Figure 7.5a and 7.5b, respectively. While diacetyl was present in higher amounts in the yogurts fermented at 0.1 MPa, higher amounts of acetoin were observed in samples fermented under pressure (10 MPa). In fact, acetoin

(M55) was identified as the metabolite more accumulated in the samples fermented at 10 MPa/43ºC (P43) and 10 MPa/50 ºC (P50) in the correspondent Volcano plots (Figure 7.4b and 7.4c, respectively).

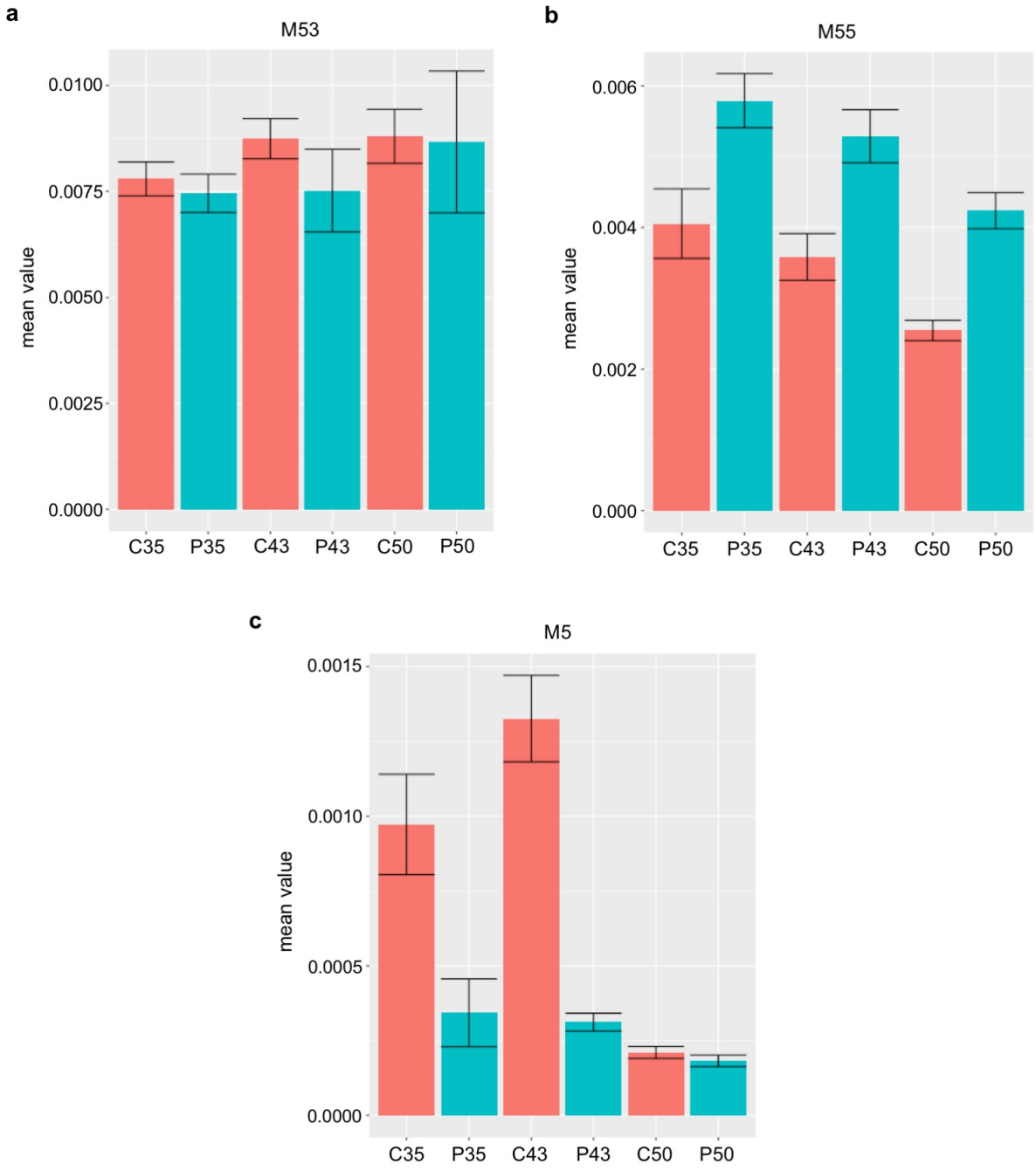

Figure 7.5. Metabolite plots showing the accumulation of diacetyl (M53, a), acetoin (M55, b), and formate (M5, c) on yogurts produced under different conditions of pressure and temperature. C35, C43 and C50 samples correspond to yogurts fermented at 0.1 MPa and 35, 43 and 50 ºC, respectively; P35, P43 and P50 samples correspond to yogurts fermented at 10 MPa and 35, 43 and 50 ºC, respectively.

Both diacetyl and acetoin are important for the typical yogurt aroma, being responsible for the butter-like flavor. For example, diacetyl contributes to the pleasant and delicate flavor and aroma of yogurt, being especially important for products with low acetaldehyde amounts (Groux, 1973; Rasic and Kurmann, 1978). On the other hand, acetoin gives a mild creamy, slightly sweet, butter-like flavor to yogurt, similarly to diacetyl. However, when comparing both flavors, acetoin had a considerably weaker flavor, usually decreasing the harshness of diacetyl (Cheng, 2010). The production of these two compounds is linked, since acetoin is the reduced form of diacetyl, produced with the irreversible action of diacetyl reductase (DR) (Collins, 1972). Therefore, the fermentation conditions used during this work apparently affect the activity of DR, with both higher pressures and lower temperatures causing an activity increase, due to the higher acetoin levels observed in these samples.

The effect of pressure on DR activity is poorly studied. However, Butz et al. (2000) observed differences on the acetoin accumulation during the ripening of Gouda cheese processed by high pressure, which they explained by variations in enzymatic activities due to pressure. Both DR and NADH oxidase can influence the acetoin accumulation: i) a higher DR activity increases the reduction of diacetyl, increasing the acetoin accumulation; and ii) a lower NADH oxidase increases the NADH available, increasing the diacetyl reduction (reaction dependent on NADH) and, as a consequence, also increasing the acetoin accumulation. Thus, despite the importance of NADH oxidase for the NAD^+ regeneration in the cells, it limits the diacetyl reduction to acetoin (Bruhn and Collins, 1970; Levata-Jovanovic and Sandine, 1996; Seitz et al., 1963). Therefore, the higher concentrations of acetoin at 10 MPa can possibly indicate that the DR activity was enhanced and/or NADH oxidase was inhibited under pressure, resulting in a higher reduction of diacetyl.

Regarding the temperature effect on these two enzymes, de Figueroa et al. (2001) observed that diacetyl and acetoin production by *Lactobacillus rhamnosus* ATCC 7469 was temperature dependent, since the activities of the enzymes involved in their production were affected by this parameter. For instance, DR activity decreased with increasing culture temperature from 22 ºC to 37 ºC, as well as NADH oxidase activity increased, resulting in a higher accumulation of diacetyl, on one hand, and lower amounts of acetoin, on the other hand. Thus, the results obtained during this work are in accordance with literature, i.e. higher diacetyl amounts in the samples fermented at higher temperatures and higher acetoin concentrations in samples fermented at lower temperatures.

Another signal showing different abundances between samples corresponded to formate, as represented in Figure 7.5c. This metabolite was even identified with the highest accumulation in samples fermented at 0.1 MPa/43 ºC (C43), accordingly to the respective Volcano plot (Figure 7.4b). Formate also influences the yogurt flavor, being characterized by a pungent flavor (Chen et al., 2017). Thus, the yogurts fermented under pressure seem to present a soft flavor when compared to control yogurts, mainly the ones fermented at 35 ºC (C35) and 43 ºC (C43). But this

compound is recognized as one of the most important factor for the associative growth of yogurt starter cultures, since it is produced by *S. thermophilus* and it stimulates the *L. bulgaricus* growth (Chandan and O'Rell, 2006; Hui et al., 2012). Accordingly to Perez et al. (1991), the production of formate by *S. thermophilus* is highly variable and dependent on several factors, including bacterial strain, culture medium and incubation temperature. The authors also observed that formate production only occurred at the late exponential and early stationary growth phases, possibly indicating that a metabolic adaptation mechanism is required to start the production. Therefore, the different fermentation conditions (both temperature and pressure) used in the current work may influence this adaptation mechanism somehow, resulting in the differences in formate abundances on the yogurts produced. Interestingly, the lower amounts of formate observed for the yogurts produced at 50 ºC (both C50 and P50) correspond to the samples with lower *S. thermophilus* counts (Chapter VI).

Additionally, other signals presented differences between samples, whether in the full spectra (Figure 7.2) or in the Volcano plots (Figure C.3). Despite these signals not being assigned, the respective abundances were determined and the obtained data is represented in Figure C.5 and C.6 in Appendix C. By analyzing the full spectra, M3, M18, M43 and M44 showed differences in the peak intensities, which were reflected in different abundances between samples (Figure C.5). For instance, M3 and M18 correspond to signals in the aromatic region, and M3 presented higher abundances in the samples fermented at 10 MPa, while M18 presented higher abundances in control samples. On the other hand, the signals identified as M43 and M44 were present in the aliphatic region and at 50 ºC (both C50 and P50) M43 was present in the greatest amount while M44 was the least concentrated. Regarding to Volcano plots, the undefined M4, M57 and M59 present significant variation between samples, with the respective abundances being also presented in Figure C.6. While M4 showed a higher abundance in the yogurts fermented at 0.1 MPa/43 ºC (C43) and 0.1 MPa/50 ºC (C50), higher amounts M57 and M59 were observed in yogurts fermented at 10 MPa/35 ºC (P35), as represented in the respective Volcano plots. Therefore, some of these signals may correspond to metabolites involved in the adaptation mechanisms of cells to both pressure and temperature, which were not fully identified and described in literature.

The spectra region assigned as correspondent to aromatic amino acids (e.g., phenylalanine, histidine, tryptophan and tyrosine, M16-M6) also presented differences between samples, as previously described. In fact, differences in the signals were detected between the yogurts fermented under pressure and at atmospheric pressure, regardless of the temperature. The normalized area of this region was also calculated (data not shown) and generally higher values were obtained for the fermentations under pressure, possibly indicating that higher amounts of free aromatic amino acids were present on these yogurts. In fact, pressure causes changes on the protein aggregation/disaggregation due to the weakening of electrostatic and hydrophobic interactions (Funtenberger et al., 1997; Heremans and Smeller, 1998), which can explain these

differences between the yogurt samples. However, these signals and the unidentified in the aromatic region can also correspond to other compounds with aromatic structures present in dairy products, such as vitamins, benzoate and orotic acid or bacteriocins (Baglio, 2014) In order to identify these metabolites, other methodology techniques must be used, including two-dimensional NMR instead of the traditional 1D ^{1}H NMR used in this work (Johanningsmeier et al., 2016).

7.4. Conclusions

Despite the minor changes between the yogurt samples tested during this work, several differences in the metabolite accumulation were observed, possibly due to the variation of the fermentation conditions (both pressure and temperature). In fact, these minimal variations may affect the sensorial properties of yogurt, since the main flavor compounds are usually present in low concentrations on yogurt. For instance, the main difference observed in this work involved the production of compounds responsible for the butter-like flavor to yogurt: diacetyl was produced in higher amounts on yogurts fermented at 0.1 MPa, while acetoin was produced in higher amounts under pressure (10 MPa), giving a softer flavor to these yogurts. In this case, the activity of both diacetyl reductase and NADH oxidase appears to be affected by the variation of pressure and temperature, resulting in these differences. However, more sample replicas should be used to confirm the significance of the differences observed between yogurts.

Overall, the effects on metabolite accumulation on yogurt may correspond to stress response mechanisms of cells in order to adapt the the new conditions of fermentation, since some of them are stressfull for microorganisms. Therefore, it would interesting to perform a transcriptomics analysis to the starter cultures to observe the changes in gene expression with the variation of the fermentation conditions. Additionally, more sensitive analytical techniques along with a broad coverage for metabolites identification should also be used to detect the minimal differences between samples and to allow the identification of the higher number of metabolites as possible, in order to disclose the influence of pressure and temperature on bacterial metabolism during growth and fermentation.

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CHAPTER VIII

Conclusions

The performance of food fermentations under non-conventional conditions has a remarkable potential to modulate the metabolic activity of microorganisms during fermentation and possibly improve the fermentative processes. During this work, both pressure and temperature was used as process variables for yogurt production and their simultaneous variation during fermentation affected not only the process kinetics, but also the final yogurt characteristics. These changes are usually translated into interesting metabolic changes on starter cultures, which, in turn, are a result of response mechanisms triggered by cells in order to be able to grow and retain their metabolic activity in the new conditions of fermentation. Thereby, the most relevant observations are summarized below:

- Sampling during fermentation under pressure could be performed through the decompression and compression of pressure vessel without any negative effect on fermentative process in what concerns to product formation rate, saving experience time and experimental resources during the work.
- Different fermentation profiles were obtained for each combination of pressure and temperature. For instance, pressure increasing slowed down yogurt fermentation, but similar fermentative profiles were achieved at 10 and 0.1 MPa at almost all temperatures tested. Regarding temperature effect, fermentation at 43 ºC presented the highest fermentative rates.
- The inhibitory effect of pressure increased when temperature decreased, with a complete inhibition of fermentation occurring at 50 MPa for 25-35 ºC, differently to 43 ºC where the complete inhibition occurred only at 100 MPa.
- Improved yields were observed at the fermentations under pressure (10 and 30 MPa) at 43 ºC, which were reflected into lactate efficiencies of 70-75 %, in contrast to 40 % at atmospheric pressure. Thus, the fermentative process showed modifications under pressure, with microorganisms more effectively converting lactose into lactate.
- Fermentation conditions affect the microbial growth of the starter cultures, with *S. thermophilus* being more sensitive to the combination of high temperatures and high pressures than *L. bulgaricus*. However, the minimum standards required to produce yogurt were achieved for almost all conditions tested.
- The physical properties of yogurts were also influenced by the variations on the fermentation conditions. The yogurts fermented at 10 MPa presented the characteristics more similar to the yogurts produced at 0.1 MPa for all temperatures tested, i.e. syneresis levels similar to control samples and a firm texture without being excessive.
- The comparative metabolomic study between the yogurts produced under different conditions showed several differences in the metabolite accumulation. The main difference involved the production of compounds responsible for the butter-like flavor to yogurt: while diacetyl was produced in higher amounts on yogurts fermented at 0.1 MPa, acetoin was

produced in higher amounts under pressure (10 MPa). Changes in the activity of both diacetyl reductase and NADH oxidase due to the variation of pressure and temperature can be behind these differences.

- Despite the changes on the butter-like flavor compounds were minimal, the variations observed can affect the sensorial perception of yogurt, since the main flavor compounds are usually present in low concentrations. Thus, yogurts produced at 10 MPa may have a softer flavor than yogurts produced at atmospheric pressure.
- Comparing the effect of pressure and temperature individually, pressure played a major role on the changes of the final yogurt properties, while temperature affect the fermentative rate at a higher extent.

Overall, the present work indicates that fermentation under non-conventional conditions can be successfully applied to food production processes, influencing the characteristics of the food products obtained. In fact, the variation of pressure and temperature during fermentation can be used to modulate these characteristics with the purpose of improving food quality and enlarging the consumer choices of the fermented product. Therefore, the studies performed during this thesis provide a general overview of the impact of varying these process variables on the final yogurts and also disclose relevant information about the adaptation of LAB to sub-lethal HP, showing that these approaches can be used to stimulate or inhibit specific metabolic pathways. The interesting potential showed during this work paves the way for further optimization studies, with the purpose of develop a HP equipment suitable for these food fermentation approaches. This will open the possibility of applying these type of strategies on a wide range of food fermentation processes, with potential to create and develop food products with novel characteristics.

APPENDIX A

Additional information supporting

CHAPTER V

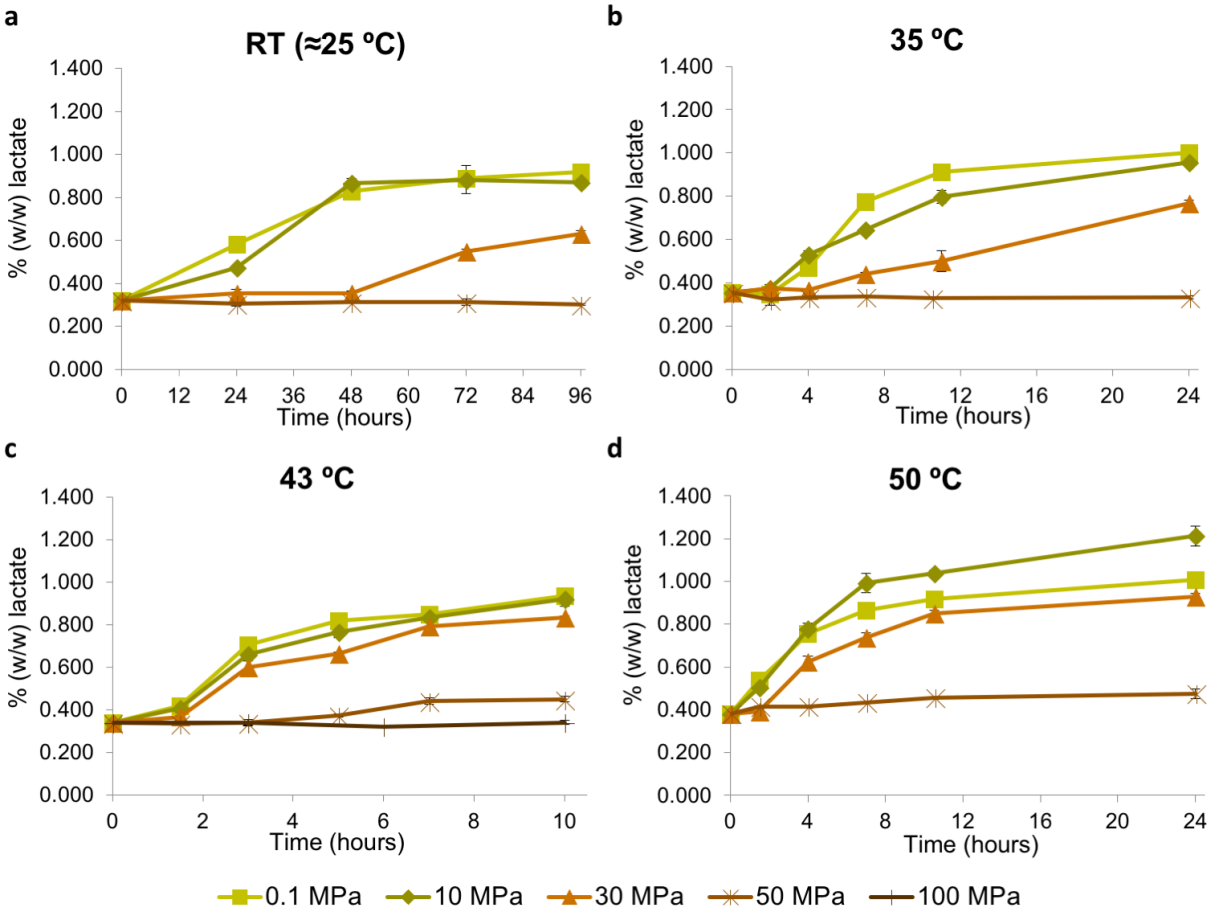


Figure A.1. Titratable acidity during fermentation at room temperature (≈25 ºC) (a), 35 ºC (b), 43 ºC (c) and 50 ºC (d), under different conditions of pressure: 10 MPa (diamonds), 30 MPa (triangles), 50 MPa (stars) and 100 MPa (crosses). Control fermentations at 0.1 MPa are represented as squares.

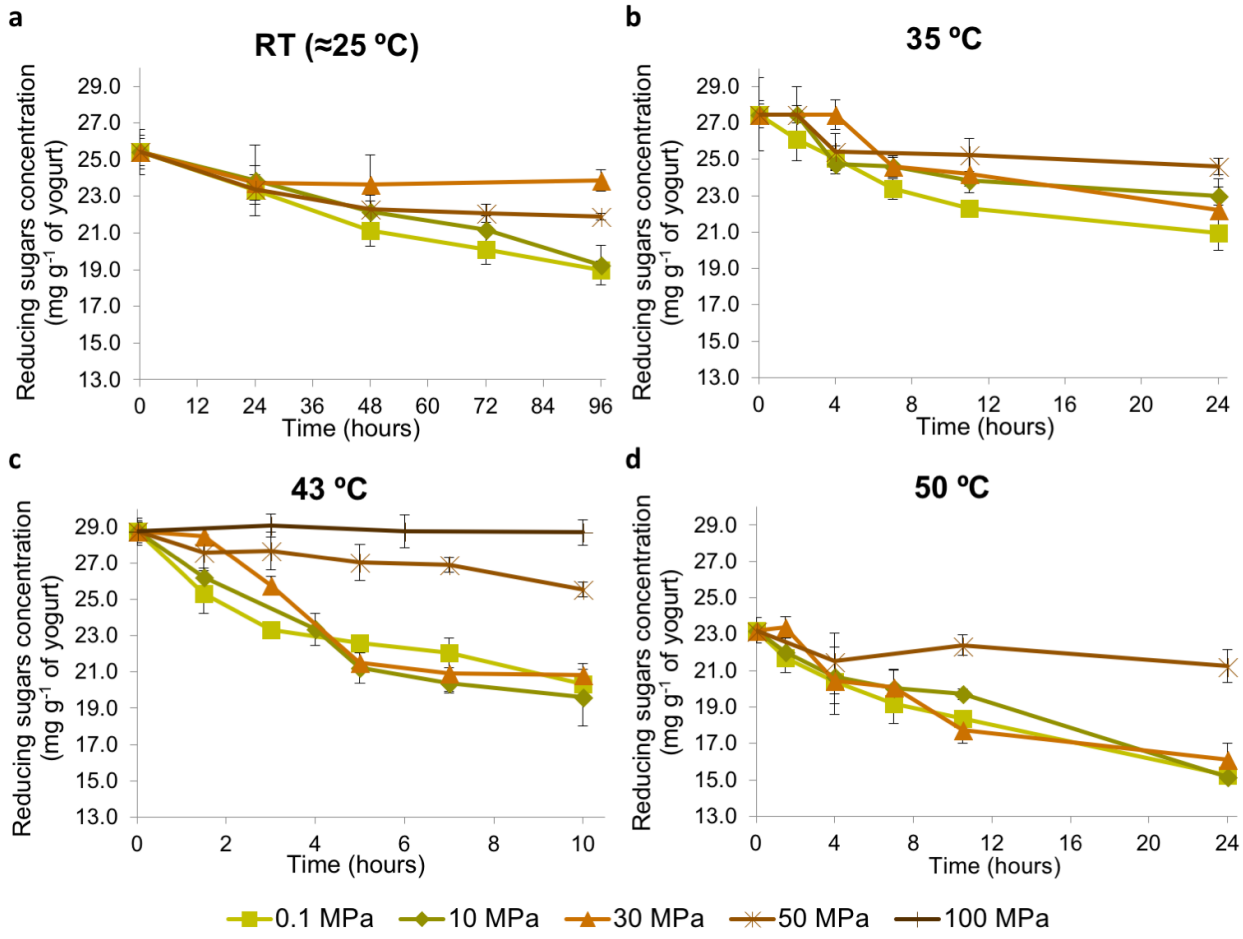


Figure A.2. Reducing sugars concentration during fermentation at room temperature (≈25 ºC) (a), 35 ºC (b), 43 ºC (c) and 50 ºC (d), under different conditions of pressure: 10 MPa (diamonds), 30 MPa (triangles), 50 MPa (stars) and 100 MPa (crosses). Control fermentations at 0.1 MPa are represented as squares.

APPENDIX B

Additional information supporting

CHAPTER VI

This appendix is based on information of the following publication:

Lopes, R.P., Mota, M.J., Pinto, C.A., Sousa, S., Gomes, A.M. Delgadillo, I., Saraiva, J.A., 2019. Physicochemical and microbial changes in yogurts produced under different conditions of pressure and temperature. LWT - Food Sci. Technol. 99, 423-430.

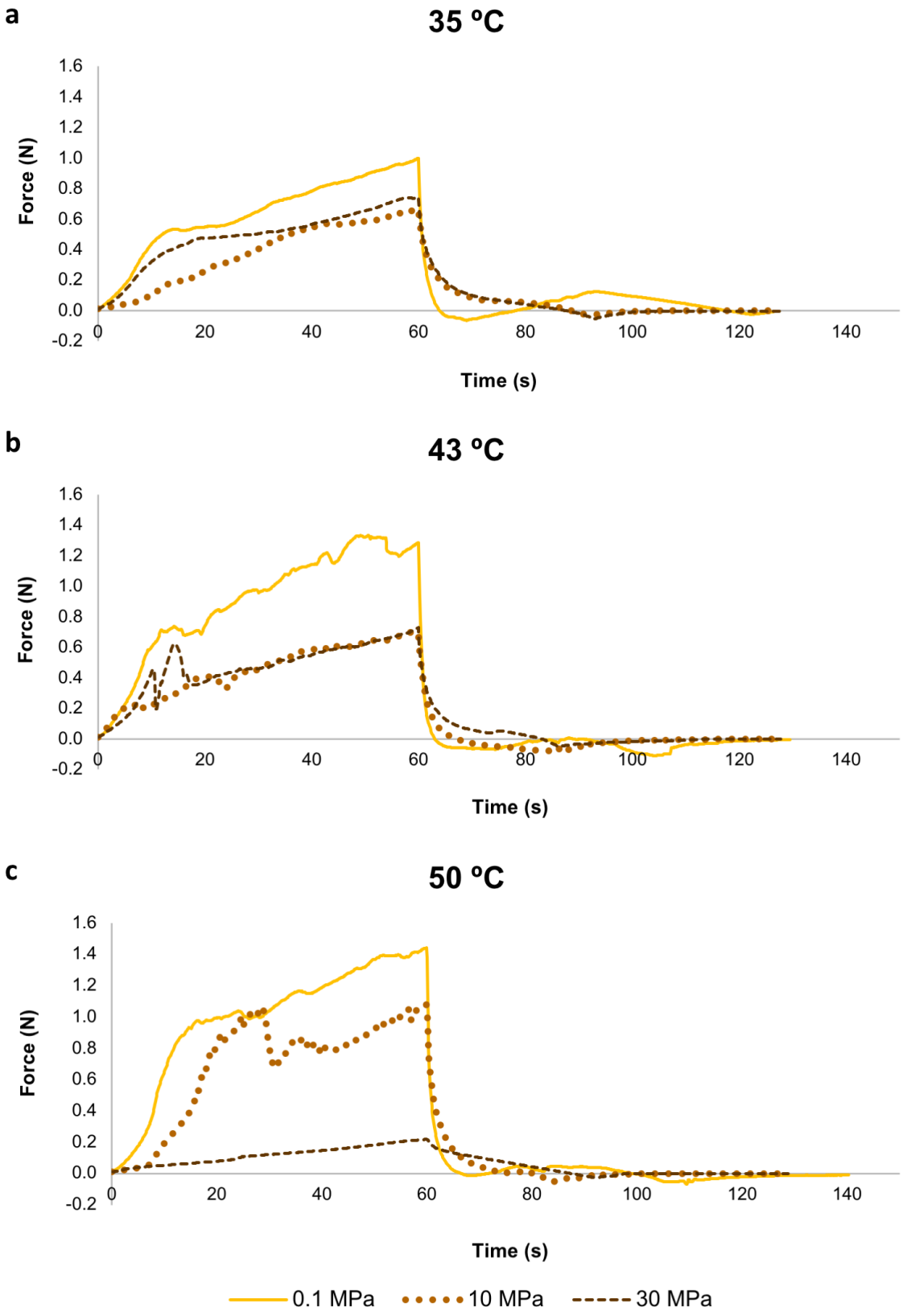


Figure B.1. Typical penetration curves (0.5 mm s^{-1}) of the yogurts produced under different pressure (0.1, 10 and 30 MPa) and temperature (35, 43 and 50 ºC) conditions.

APPENDIX C

Additional information supporting

CHAPTER VII

Table C.1. List of the peaks present in the samples analyzed, with the code name assigned (attributed between M1 and M66, from higher to lower ppm values) and the respective chemical shifts.

Code name	Chemical shift (ppm)	Code name	Chemical shift (ppm)
M1	8.57 – 8.59	M34	4.44 – 4.49
M2	8.54 – 8.57	M35	4.41 – 4.43
M3	8.50 – 8.54	M36	4.11 – 4.17
M4	8.46 – 8.48	M37	4.07 – 4.11
M5	8.44 – 8.46	M38	3.46 – 4.00
M6	7.86 – 7.90	M39	3.26 – 3.34
M7	7.82 – 7.86	M40	3.24 – 3.26
M8	7.62 – 7.65	M41	3.22 – 3.24
M9	7.53 – 7.59	M42	3.19 – 3.22
M10	7.47 – 7.52	M43	3.06 – 3.08
M11	7.41 – 7.46	M44	3.03 – 3.05
M12	7.35 – 7.40	M45	2.99 – 3.03
M13	7.30 – 7.35	M46	2.84 – 2.86
M14	7.16 – 7.21	M47	2.81 – 2.84
M15	6.88 – 6.93	M48	2.79 – 2.81
M16	6.84 – 6.88	M49	2.67 – 2.75
M17	6.62 – 6.65	M50	2.50 – 2.58
M18	6.51 – 6.54	M51	2.45 – 2.49
M19	6.18 – 6.22	M52	2.42 – 2.45
M20	6.12 – 6.16	M53	2.37 – 2.38
M21	5.94 – 6.01	M54	2.33 – 2.37
M22	5.50 – 5.55	M55	2.22 – 2.24
M23	5.35 – 5.44	M56	2.16 – 2.22
M24	5.28 – 5.31	M57	2.13 – 2.15
M25	5.26 – 5.28	M58	2.03 – 2.08
M26	5.22 – 5.25	M59	1.98 – 2.02
M27	5.19 – 5.21	M60	1.92 – 1.95
M28	5.08 – 5.11	M61	1.48 – 1.50
M29	5.05 – 5.08	M62	1.44 – 1.48
M30	4.73 – 4.84	M63	1.28 – 1.40
M31	4.66 – 4.70	M64	1.23 – 1.24
M32	4.64 – 4.66	M65	1.19 – 1.22
M33	4.58 – 4.61	M66	1.12 – 1.16

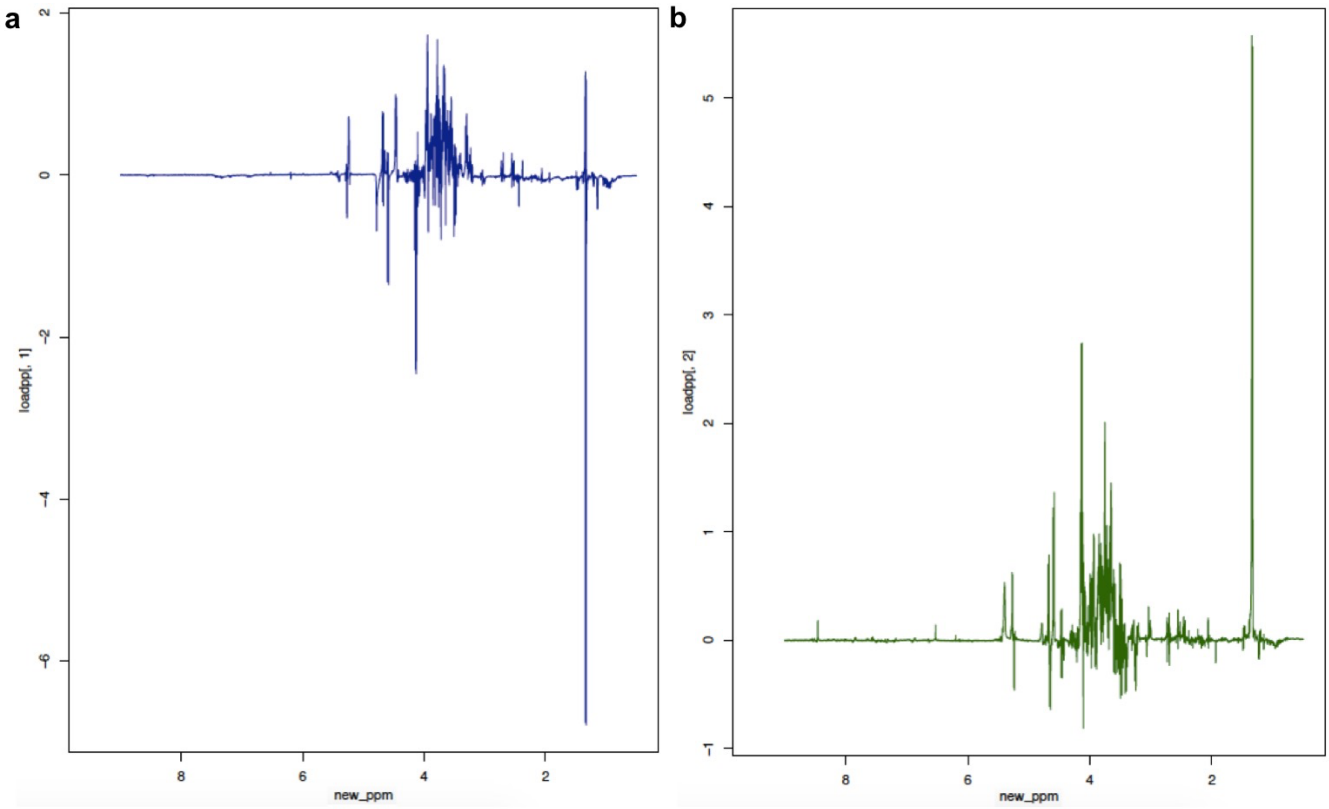


Figure C.1. Principal component analysis (PCA) loading plots 1 (a) and 2 (b) of yogurt produced under different conditions of pressure and temperature, obtained by 1D ^{1}H NMR.

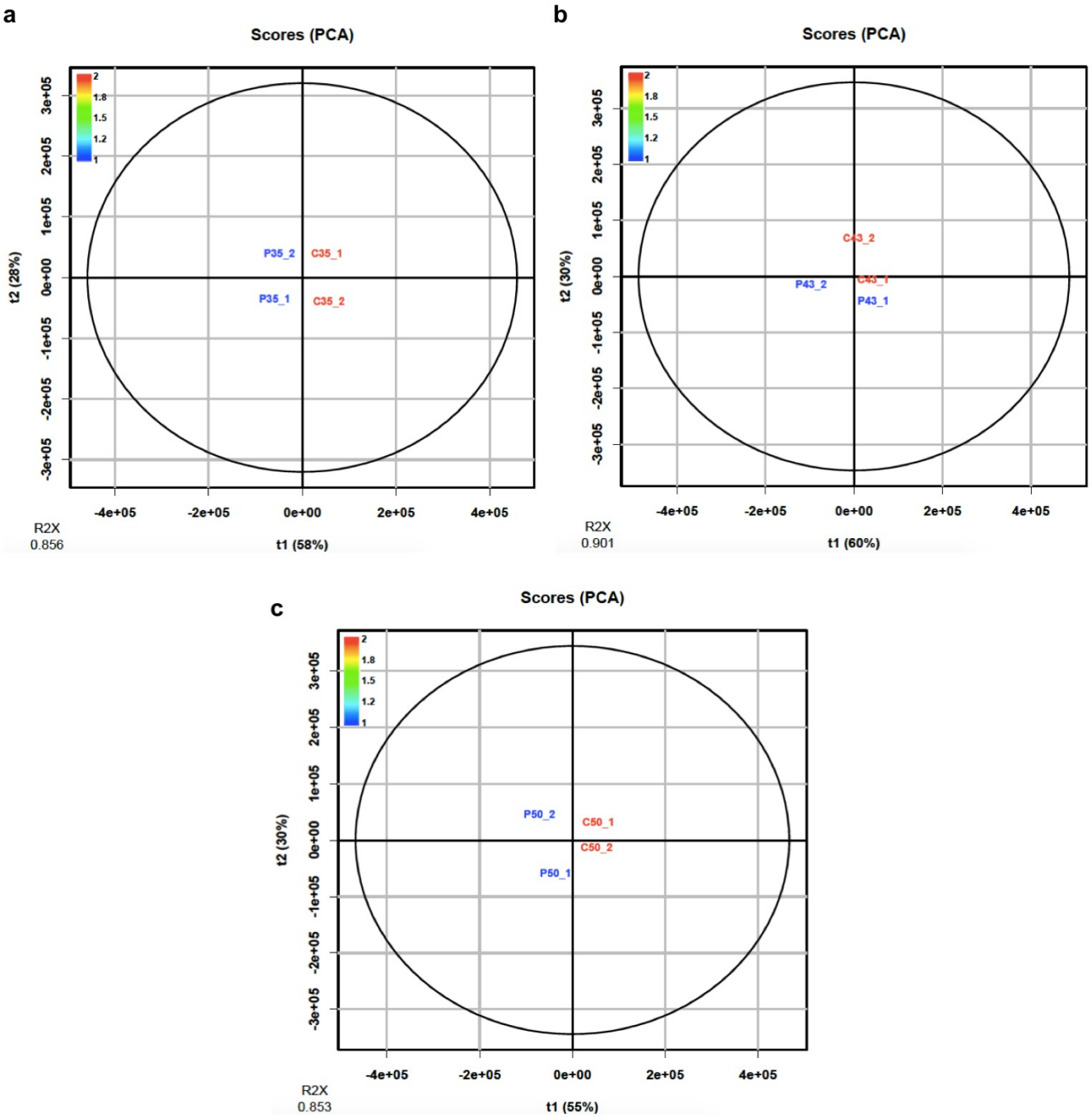
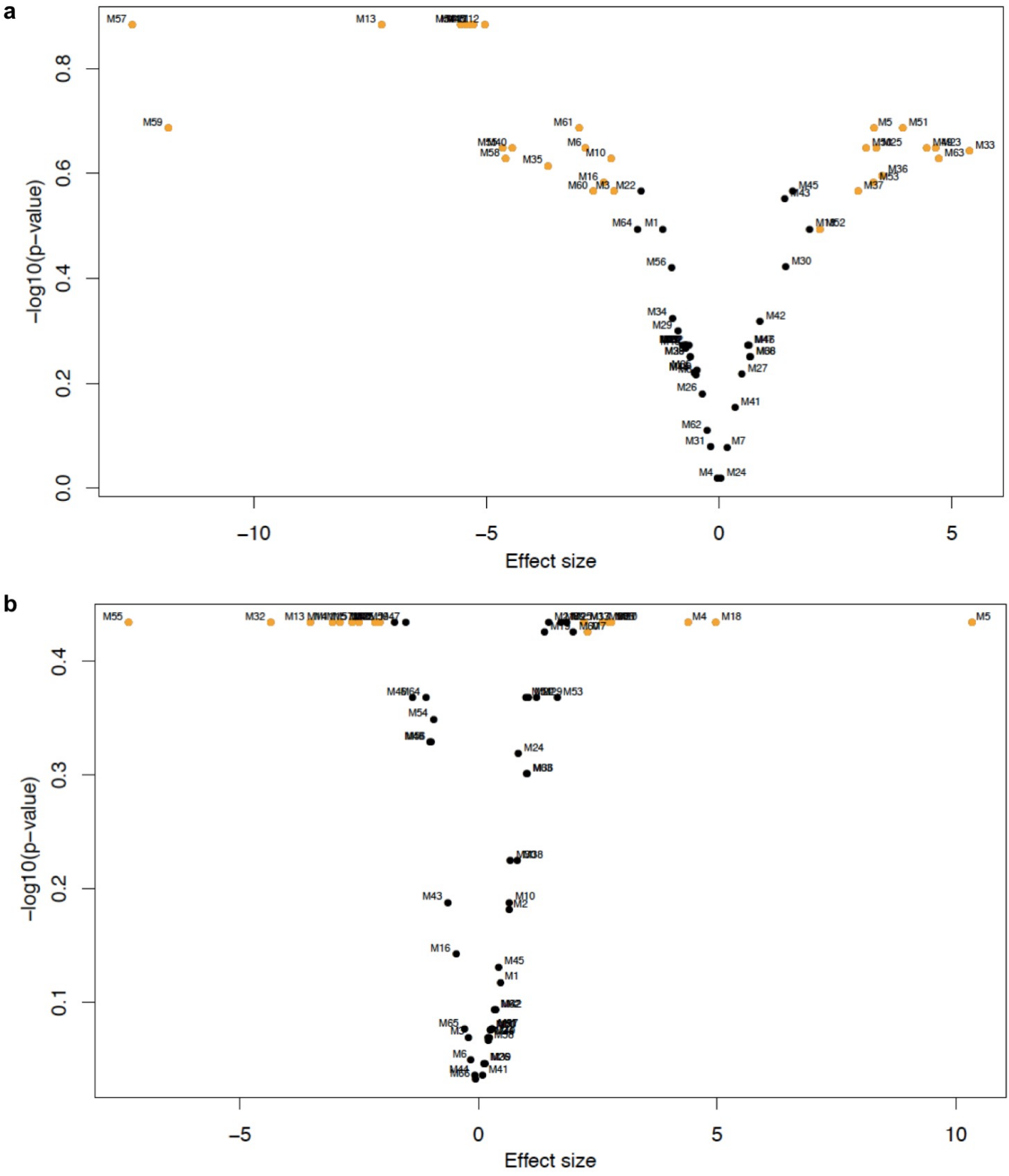


Figure C.2. PCA scores plot of yogurt produced under pressure (10 MPa, P samples) and at atmospheric pressure (0.1 MPa, C samples), for each temperature tested: 35 ºC (a), 43 ºC (b) and 50 ºC (c), obtained by 1D ^{1}H NMR. C35, C43 and C50 samples correspond to yogurts fermented at 0.1 MPa and 35, 43 and 50 ºC, respectively; P35, P43 and P50 samples correspond to yogurts fermented at 10 MPa and 35, 43 and 50 ºC, respectively. In all cases, the results are presented in duplicated (indicated as 1 and 2).


a
−log10(p−value)
Effect size
b
−log10(p−value)
Effect size

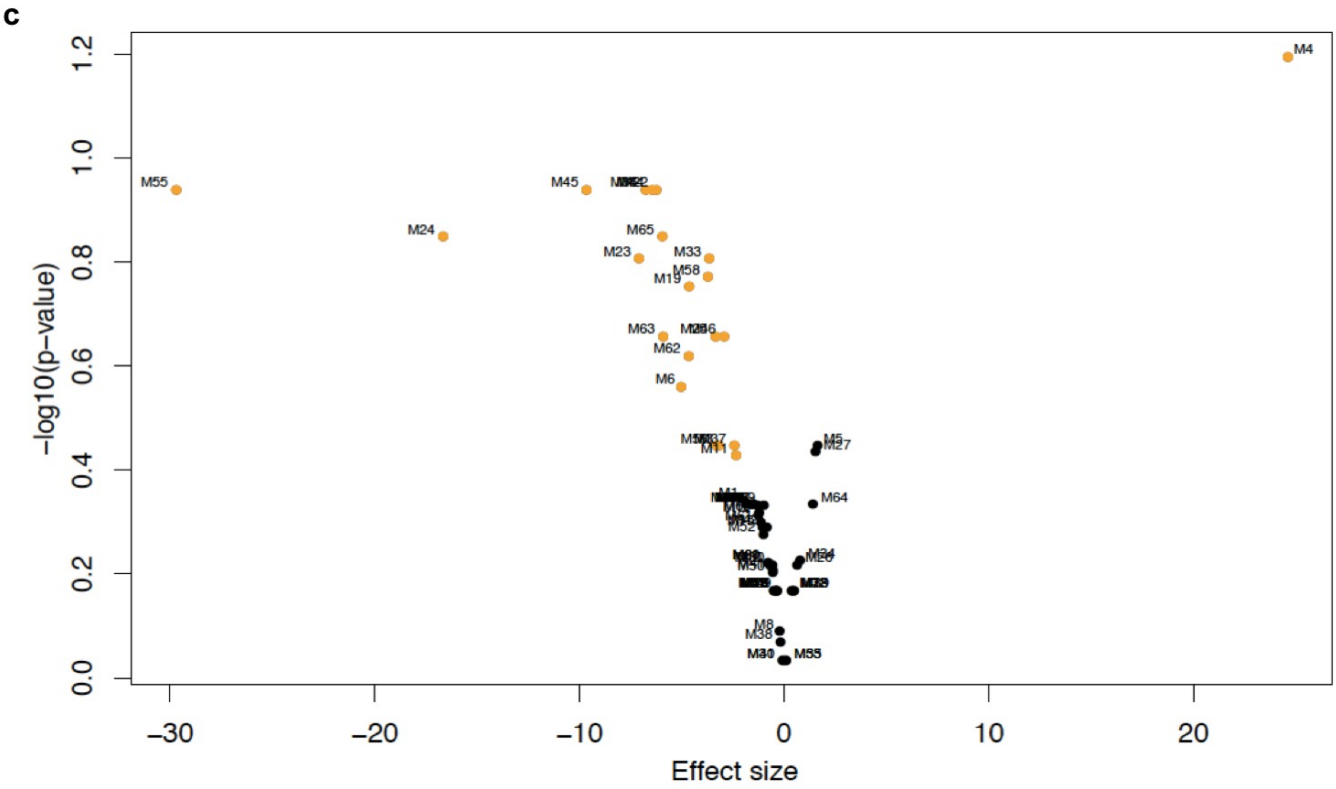


Figure C.3. Volcano plots showing the differences in the metabolic profiles of yogurt produced under pressure (10 MPa) and at atmospheric pressure, for each temperature tested: 35 ºC (a), 43 ºC (b) and 50 ºC (c). The signals/metabolites are indicated by a code name, between M1 and M66. The x-axis represents the effect sizes (plotted on a log 2 scale) of the relative abundance of each metabolite between the samples fermented at 10 and 0.1 MPa. The y-axis represents the statistical significance p-value of the ratio fold-change for each metabolite. Metabolites whose abundance is unchanged between the two samples will plot at the x-axis origin. Metabolites that hyper-accumulate in one of the two samples under analysis will plot either to the left (10 MPa) or right (0.1 MPa) of the x-axis origin.

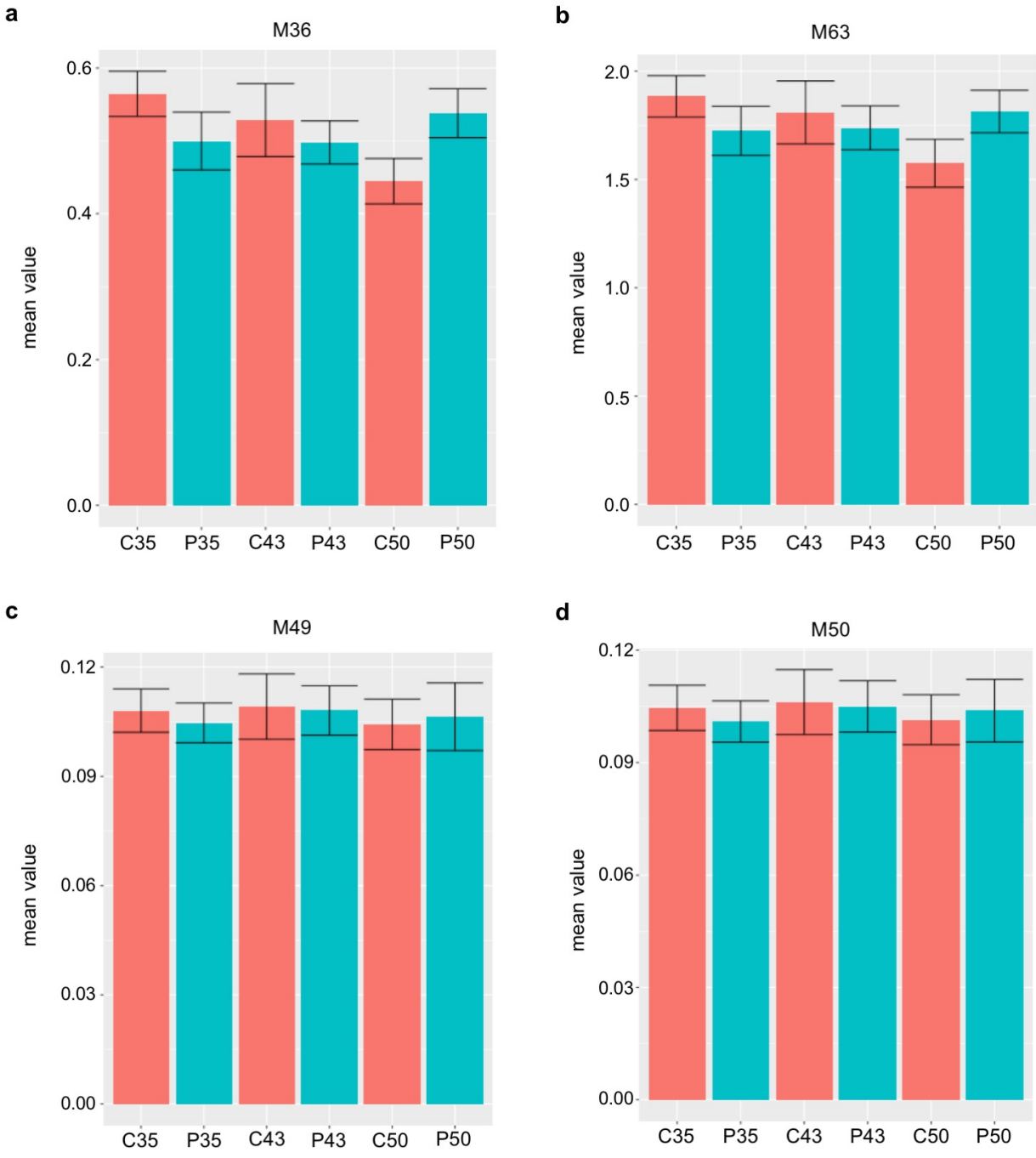


Figure C.4. Metabolite plots showing the accumulation of lactate (M36 and M63, a and b, respectively) and citrate (M49 and M50, c and d, respectively) on yogurts produced under different conditions of pressure and temperature. C35, C43 and C50 samples correspond to yogurts fermented at 0.1 MPa and 35, 43 and 50 ºC, respectively; P35, P43 and P50 samples correspond to yogurts fermented at 10 MPa and 35, 43 and 50 ºC, respectively.

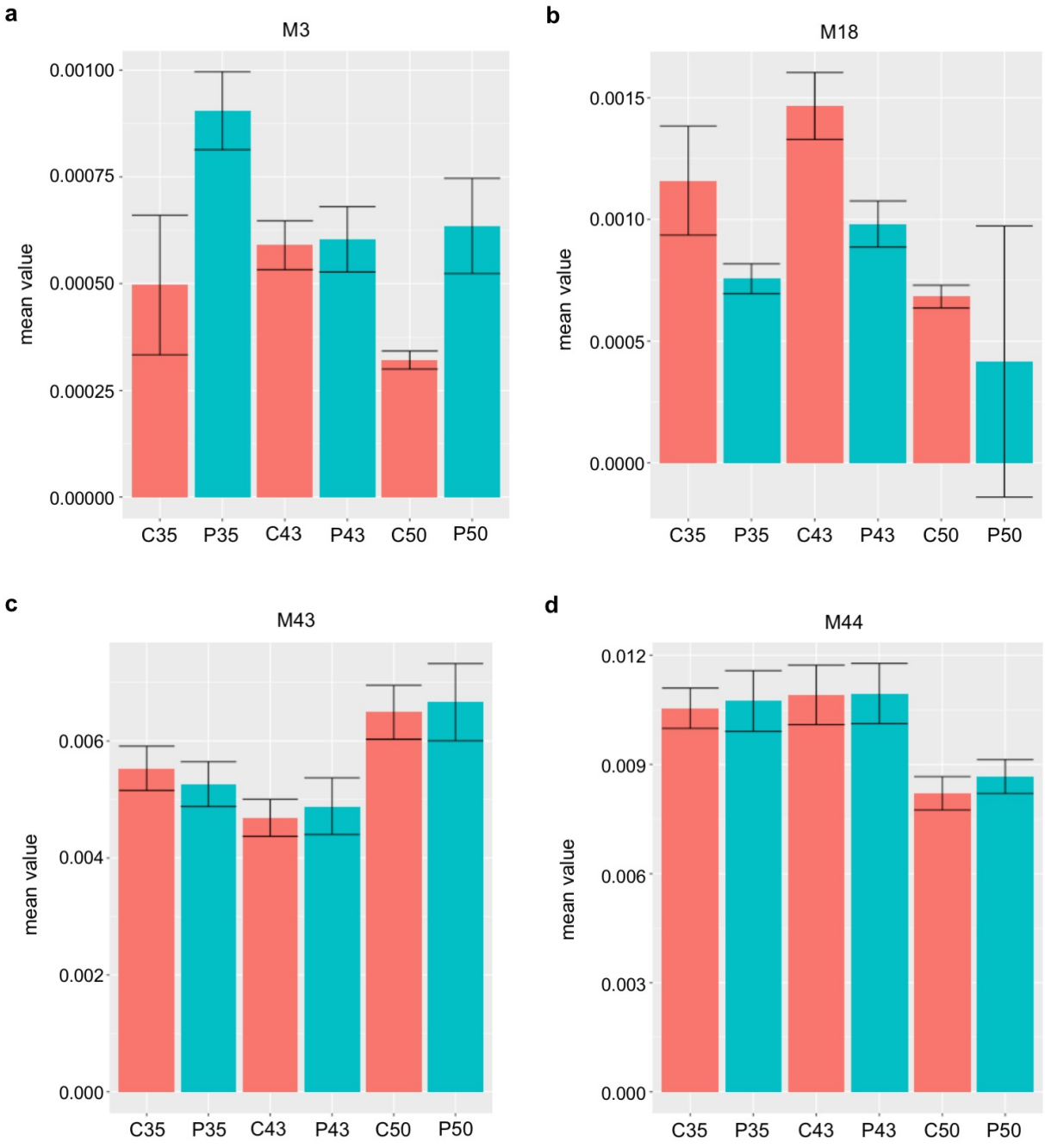


Figure C.5. Metabolite plots showing the accumulation of the unidentified signals M3, M18, M43 and M44, that presented visible differences in full spectra between yogurts produced under different conditions of pressure and temperature. C35, C43 and C50 samples correspond to yogurts fermented at 0.1 MPa and 35, 43 and 50 ºC, respectively; P35, P43 and P50 samples correspond to yogurts fermented at 10 MPa and 35, 43 and 50 ºC, respectively.

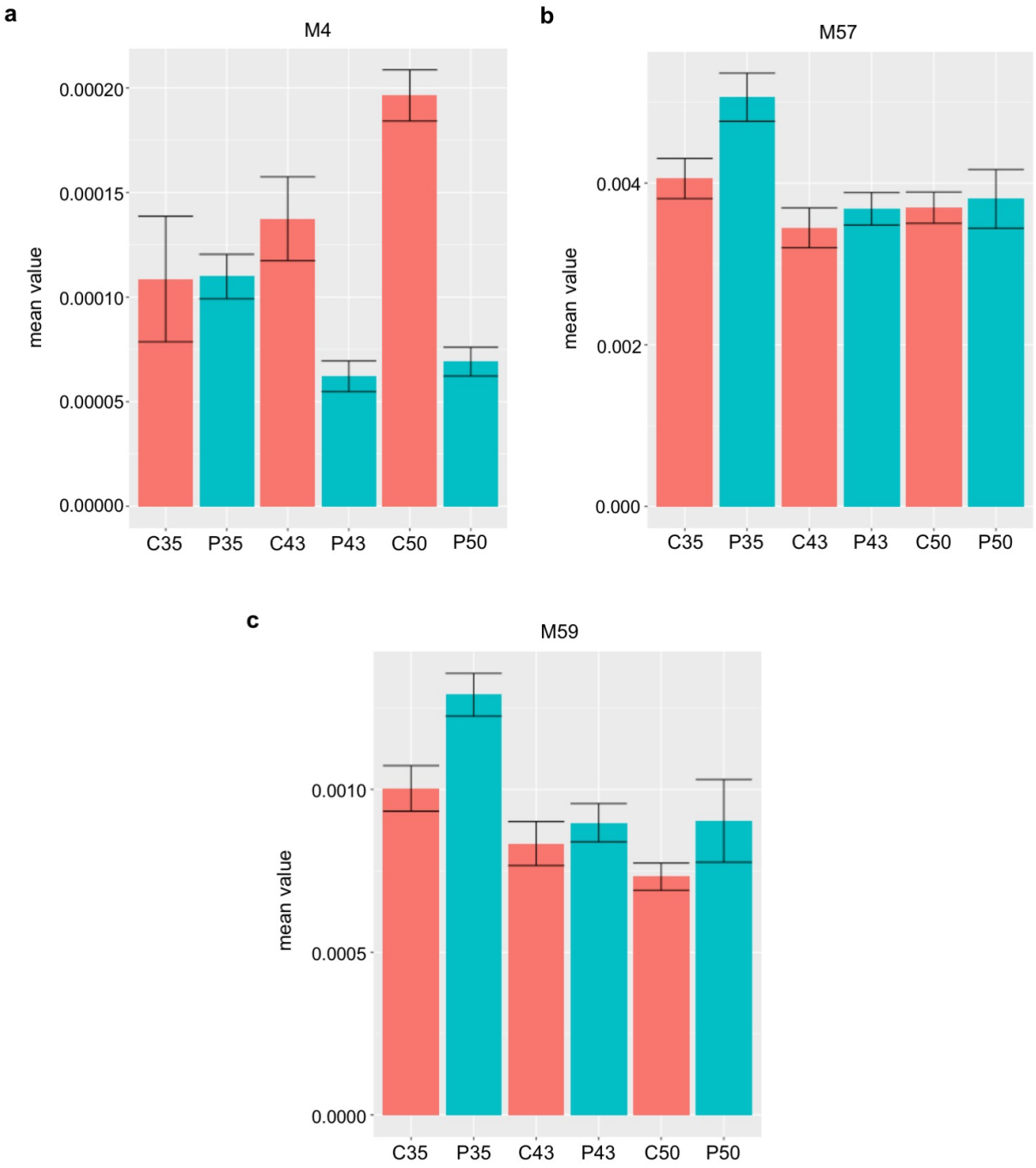


Figure C.6. Metabolite plots showing the accumulation of the unidentified signals M4, M57 and M59, that presented significant differences in Volcano plots between yogurts produced under different conditions of pressure and temperature. C35, C43 and C50 samples correspond to yogurts fermented at 0.1 MPa and 35, 43 and 50 ºC, respectively; P35, P43 and P50 samples correspond to yogurts fermented at 10 MPa and 35, 43 and 50 ºC, respectively.